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# UPTAKE OF MANNITOL INTO THE SHOOTS OF INTACT BARLEY PLANTS

By H. GROENEWEGEN\* and J. A. MILLS†

[Manuscript received September 7, 1959]

## Summary

Barley plants grown in mannitol solutions wilted much more severely than those grown in sodium chloride solutions. Recovery from wilting after transference to a basal nutrient solution was rapid.

Following transference to a basal nutrient solution mannitol was excreted from the leaves in the guttation water.

The physiological importance of this uptake of mannitol is briefly discussed.

## I. INTRODUCTION

Mannitol is used frequently as an osmotic substance in short-term experiments on water relations of plant tissues. For such experiments it is presumed to be physiologically inert, and its permeation is considered to be very slow (Collander and Bärlund 1933; Allsopp 1955). Collander and Bärlund studied the permeation of non-electrolytes in the cell sap of *Chara ceratophylla*. They assessed the half saturation time‡ for mannitol to be greater than 35 days.

The use over a period of some days of an osmotic substance not absorbed by the plant would be advantageous in work on water relations of intact plants and in salinity studies. In salinity studies the use of mannitol could help to distinguish between osmotic effects, caused by the reduced water availability in the substrate, and effects of ions absorbed by the plant.

Such a comparison between the effects of sodium chloride and mannitol on the growth of young barley plants was made. In this paper evidence is presented that mannitol was absorbed during the course of the experiment by the shoots of the intact plants.

## II. METHODS

The results described in this paper were obtained in an experiment which was part of an investigation regarding salinity effects on plant growth. In this work plants are subjected to a brief salinity stress. After subsequent salt removal from the substrate a study is made of the recovery in growth.

Barley seeds, cv. Chevron, were germinated in river sand. When the first leaf had fully expanded the seedlings were transplanted into basal nutrient solution of Arnon (cf. Hewitt 1952). This solution had a pH of 4.5, and 1 m-equiv/l of sodium chloride was supplied.

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‡ The half saturation time is defined as the time in which the concentration of the cell sap had attained 50 per cent. of the concentration of the substrate.

Treatments were applied at the sixth day after transplanting. They consisted of basal nutrient solution, and of the same solution to which either mannitol or sodium chloride was added. In the mannitol and sodium chloride treatments the osmotic pressure of the solution was increased each day by 2 atm. The highest concentration (8 atm) was attained at the fourth day of the treatment. The plants were then transferred to basal nutrient solution on the morning of the fifth day after treatment application.

To prevent fungal development in the mannitol solutions the roots of all treatments were washed thoroughly each day with half a litre of the appropriate solution. This rinsing and replacement of solution was also carried out 2 and 4 hr after mannitol removal, and then again each morning. No noticeable fungal development took place during the experiment.

Twelve replicates of each treatment were harvested on three occasions: at treatment application ( $H_1$ ), at date of mannitol removal ( $H_2$ ), and 4 days later ( $H_3$ ).

Mannitol was detected by the paper-ionophoresis method of Frahn and Mills (1959).

### III. RESULTS

#### (a) *Growth and Wilting Response*

The dry weight and relative growth rate of the mannitol-treated plants was depressed during the mannitol treatment and also, more strongly, in the period following mannitol removal (Table 1). Slight wilting was observed in the mannitol-treated plants after the concentration was increased to 4 atm. Wilting phenomena in solutions with an osmotic pressure of 8 atm are shown in Plate 1, Figure 1. All the leaves of mannitol-treated plants wilted severely directly after transference to the 8 atm solution. At this concentration only the youngest leaf of the sodium chloride-treated plants showed slight wilting. After mannitol and sodium chloride removal all plants recovered rapidly.

The wilting in the mannitol cultures was presumably mainly due to the high osmotic pressure of the substrate. This was indicated by the sudden increase in wilting phenomena which was observed directly after an increase in the concentration of the solution. It is of interest that sodium chloride solutions of the same osmotic strength showed much later and less severe wilting.

#### (b) *Excretion of Crystalline Substance*

Mannitol-treated plants showed a necrotic burn of the leaf tips at the date of mannitol removal. This burn extended, in the old leaves, about 1–1½ cm from the tip.

During the night following mannitol removal, all mannitol and control plants guttated vigorously. The following morning a white crystalline substance was found on all the leaves of the mannitol-treated plants. The substance was usually found on the leaf tips (Plate 1, Fig. 2), but sometimes guttation water fell either on the cardboard covers or on the leaf blades. In all cases the crystalline substance was found in these locations, causing necrotic burns in the case of the leaf blades. The substance was collected from the leaf tips only.



(c) *Nature of the Crystalline Substance*

The solid left on evaporation of the guttation water was dissolved in water to a concentration of 35 mg/ml. This solution was used for paper ionophoresis in borax at pH 9.2, sodium arsenite at pH 9.6, and basic lead acetate (Frahn and Mills 1959). In each electrolyte, the unknown afforded only one component, migrating at the same rate, and reacting in the same way with spray reagents as pure D-mannitol run on the same paper strips. The substance had m.p. 164°C, not depressed by mixing it with pure D-mannitol of m.p. 166°C. Condensation of 2 mg of the solid with excess cyclohexanone containing sulphuric acid gave, in 55 per cent. yield, a crystalline

TABLE 1  
DRY WEIGHT AND RELATIVE GROWTH RATE OF WHOLE BARLEY PLANT

Treatment	Dry Weight (g)			Relative Growth Rate (g/g/day)	
	At Treatment Application (H <sub>1</sub> )	At Date of Mannitol Removal (H <sub>2</sub> )	4 Days after Mannitol Removal (H <sub>3</sub> )	H <sub>1</sub> -H <sub>2</sub>	H <sub>2</sub> -H <sub>3</sub>
Control	0.0471	0.083	0.155	0.142	0.139
Mannitol	—	0.078	0.120	0.126	0.108
Sodium chloride	—	0.079	0.126	0.130	0.115

derivative, shown by melting point and mixed melting point determinations to be identical with 1,2:3,4:5,6-tri-*O*-cyclohexylidene-D-mannitol (Bourne, Corbett, and Erilinne 1950). This derivative is very suitable for the chemical identification of small quantities of D-mannitol.

These results showed that the crystalline substance in the guttation water was essentially pure D-mannitol.

## IV. DISCUSSION

The excretion of the mannitol in the guttation water is, as far as the authors are aware, the first direct evidence in the literature of mannitol absorption by the shoots of intact plants.

Some workers have presented evidence for mannitol absorption by the vacuole, in addition to the normal permeation into the free space of roots and tissue disks. Ordin, Applewhite, and Bonner (1956) found that the osmotic pressure of sections of the *Avena* coleoptile, after immersion for 20 hr in a mannitol solution, had increased slightly. Since some elongation of the tissue had taken place they concluded that a small amount of mannitol must have entered the vacuoles of the tissue. Burström

(1953) found increases in the osmotic pressure of the cell sap after immersion of disks of *Helianthus* tuber for 54 hr in mannitol solution and concluded that a substantial amount of mannitol had been absorbed by the vacuoles. Direct evidence for a mannitol permeation into the vacuole was presented by Collander and Bårlund (1933).

The data presented in this paper show that mannitol molecules are not excluded from the shoots of intact plants. It should be remembered, however, that solutions having high osmotic pressures were used in this experiment. It is possible that the roots were damaged by these concentrations, so that other results might have been obtained at lower mannitol concentrations.

It is relevant to consider the implications of the presence of mannitol in the shoots of intact plants. The absorbed mannitol might obviously have contributed significantly to the fairly small dry weight increases of the present experiment. The relative growth rates both before and after mannitol removal would be affected. That for  $H_1-H_2$  would be increased and that for  $H_2-H_3$  decreased.

Of more importance are the physiological implications of the presence of mannitol in the shoots of the intact plants. In studies of plant-water relations the increase of the osmotic pressure of the cell sap would be of relevance. The mannitol may be, moreover, variably distributed within the plant. For example, the presence of mannitol in the guttation water shows that mannitol must have been present in the xylem. It might, on the other hand, have been entirely excluded from the vacuoles. The presence of mannitol could have wider physiological implications which, however, it is not relevant to discuss here.

It can be concluded that mannitol is not a suitable substance to impose a water stress on intact plants for any considerable length of time.

## V. ACKNOWLEDGMENTS

The authors are indebted to Mr. T. Mitchell, Irrigation Research Station, C.S.I.R.O., Griffith, for assistance during the experiment and to Mr. J. Miller, Irrigation Research and Extension Committee, Griffith, for the photographic work.

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MANNITOL UPTAKE IN BARLEY PLANTS



Fig. 1.—Wilting phenomena in mannitol- and sodium chloride-treated barley plants (osmotic pressure 8 atm).



Fig. 2.—Crystalline substance on tip of barley leaf 24 hr after mannitol removal.



# MEMBRANE STABILITY IN *MICROCOCCUS LYSODEIKTICUS* AND ITS PROTOPLASTS

By A. R. GILBY\* and THE LATE A. V. FEW†

[Manuscript received September 15, 1959]

## Summary

The response of *Micrococcus lysodeikticus* and its protoplasts to storage in 1M sucrose has been studied by spectrophotometry and electron-microscopy. During several days, changes in membrane permeability properties result in the leakage of intracellular components absorbing at 260 m $\mu$  without loss of the stabilizing effect of sucrose against osmotic explosion. Under some conditions of temperature and pH, autolytic conversion of bacteria to osmotically sensitive "protoplasts" can be almost complete. Fresh protoplasts in 0.05M NaCl plus 1M sucrose are stable within the range of pH 5.5–8.0. Exposure of protoplasts to pH conditions outside these limits causes lysis with disruption of the protoplast membrane.

## I. INTRODUCTION

The well-known lytic effect of lysozyme on *Micrococcus lysodeikticus* (Fleming 1922) by depolymerizing its cell wall (Salton 1952, 1956) has led to a number of attempts to prepare protoplasts from this organism by adapting the methods used successfully by Weibull (1953) with *Bacillus megaterium*. Due to the much higher osmotic pressures necessary in the stabilizing medium during the controlled action with lysozyme, some of these attempts were only partially successful with *M. lysodeikticus*. However, McQuillen (1955a) and Mitchell and Moyle (1956a, 1956b) have now described the liberation by lysozyme of protoplasts of *M. lysodeikticus* and have studied some of their properties. In other publications (Few, Fraser, and Gilby 1957; Gilby and Few 1957a, 1957b) the results of experiments with stable preparations in sucrose of protoplasts from *M. lysodeikticus* have been reported.

Our work on the preparation of protoplasts of *M. lysodeikticus* (Gilby 1957) has confirmed that of Mitchell and Moyle referred to above. Protoplasts were prepared by treatment with lysozyme in media containing sucrose at different concentrations. Measurements are made of the optical density at 500 m $\mu$  of protoplast suspensions and of the ultraviolet absorption spectra of the supernatants from suspensions which had been centrifuged with precautions to avoid mechanical damage (Weibull 1953). Both types of measurement indicated that 95 per cent. of protoplasts were stabilized when prepared in 1M sucrose and no improvement was achieved at higher concentrations.

Thus, provided lysozyme treatment is carried out in the presence of 1M sucrose, there appears to be insignificant damage to the permeability barriers of

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*M. lysodeikticus*. This paper describes subsequent changes occurring on storage of protoplasts and of bacteria and the stability of the protoplast membrane to pH.

## II. METHODS

### (a) *Growth of Bacteria and Preparation of Protoplasts*

*M. lysodeikticus* (N.C.T.C. 2665) was grown in surface culture on a beef tryptic digest medium at pH 7.2 contained in 20-oz bottles which were plugged with cotton wool. Each bottle was inoculated with *c.* 2 ml of a 48-hr broth culture. After incubation for 20 hr at 37°C the organisms were harvested, passed through a coarse glass-wool filter, and washed three times by centrifugation with distilled water. Finally they were resuspended in distilled water at a concentration of *c.* 30 mg dry wt./ml. To prepare protoplasts, bacteria at a concentration of 10 mg dry wt./ml were treated with lysozyme (concn. 100 µg/ml) in a medium containing 1M sucrose plus 0.05M NaCl. As indicated by spectrophotometric measurements at 500 mµ, depolymerization of the cell walls was complete after 30 min at 20°C. After this time, the protoplast suspension was centrifuged at *c.* 1200 *g* for 30 min and the supernatant reaction medium, containing 10–15 per cent. of the protoplasts still unsedimented, discarded. The sedimented pellet was rinsed with the sucrose medium and the protoplasts were gently resuspended in 1M sucrose plus 0.05M NaCl. In this way the complex mixture of reaction products was removed without the protoplasts undergoing any destruction detectable by phase- or electron-microscopy or by spectrophotometry.

### (b) *Senescence of Bacteria and Protoplasts*

Stock suspensions of bacteria and of protoplasts were prepared at 5 mg bacterial dry wt./ml. Two types of suspension medium were used, each containing 1M sucrose, but one was buffered at pH 6.5 and the other at pH 5.5 with 0.05M phosphate buffer. The organisms were stored at 15°C and at 0°C. At intervals over 6 days, 1-ml samples were withdrawn and each diluted with 4 ml of the appropriate sucrose medium. The optical density at 500 mµ of each of the diluted suspensions was determined before centrifugation. Centrifugation was carried out in four stages to minimize mechanical damage to abnormally sensitive organisms. These stages consisted in two centrifugations at 400–500 *g* for 10 min followed by another at 1500 *g* for 30 min, each at 2°C, and finally centrifugation at 3000 *g* for 30 min at room temperature. The optical density at 260 mµ was then measured on the clear supernatants. Spectrophotometric determinations were made using a Unicam SP500 spectrophotometer.

Observations with the electron microscope were made on bacteria and protoplasts before and after aging. The organisms, at a concentration of 2 mg dry wt./ml, were fixed by treatment for 30 min at room temperature with 2 per cent. formalin in 1M sucrose. Osmic acid treatment was not successful as this caused lysis of protoplasts. The formalin-hardened preparations were centrifuged and washed twice in distilled water. The specimens were dried under vacuum after transfer to nitrocellulose-filmed grids and shadowed with gold-palladium before examination in a Siemens electron microscope, generally at a direct magnification of 8000–12,000.



## (c) pH Stability of Protoplasts

It was found impossible to centrifuge protoplasts which had been exposed to unfavourable pH conditions without additional uncontrolled mechanical damage. Calibration curves were constructed relating the optical density at 500 and 260  $m\mu$  to the composition of artificially prepared mixtures of protoplasts and protoplasts

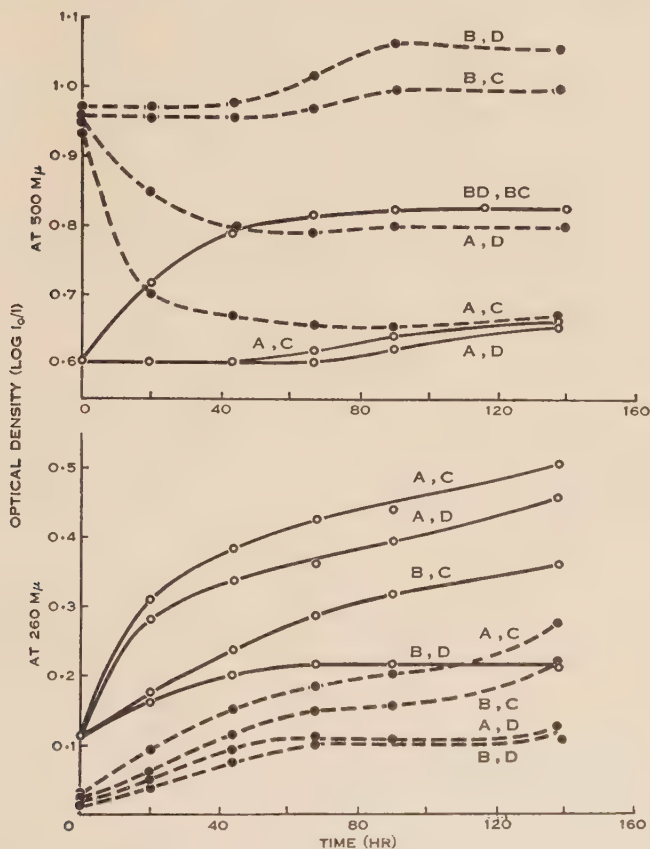


Fig. 1.—Storage of *M. lysodeikticus* (broken lines) and protoplasts at a concentration of 1 mg dry wt./ml in 1M sucrose. Optical densities at 500  $m\mu$  measured on suspensions and at 260  $m\mu$  on centrifuged supernatants. Conditions of storage indicated on curves:

A, pH 6.5; B, pH 5.5; C, 15°C; D, 0°C.

lysed by osmotic explosion. These mixtures corresponded to a total dry weight of 0.25 mg/ml and were made up in 1M sucrose plus 0.05M NaCl, and ranged from 100 per cent. protoplasts to 100 per cent. lysate.

To determine their pH sensitivity, protoplasts at a concentration of 2.5 mg dry wt./ml were suspended in 1M sucrose buffered to cover the range of pH 4–11.5. The buffers were used at an approximate ionic strength of 0.05 and consisted of mixtures of citric acid and  $\text{Na}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$ ,  $\text{Na}_2\text{B}_4\text{O}_7$  and NaOH.

Samples were taken after 0.5 and 2.5 hr at the test pH and returned to pH 6.5 by a dilution of 1/10 into 1M sucrose buffered at this pH. This was to avoid the complication caused by precipitation at low pH of the cell contents of lysed protoplasts. The optical density at 500 and 260  $m\mu$  were then determined for each of the diluted suspensions.

### III. RESULTS

#### (a) *Effects of Storage*

The results of measurements of optical density at 500 and 260  $m\mu$  on suspensions of bacteria and protoplasts stored under various conditions are illustrated in Figure 1. Appreciable changes occur with time in each case. The 260  $m\mu$

TABLE 1  
OPTICAL DENSITIES OF 1/10 DILUTIONS OF BACTERIA STORED IN 1M SUCROSE  
FOR 6 DAYS  
Final concentrations 0.5 mg dry wt./ml, 0.1M sucrose

Conditions of Storage		Optical Density at 500 $m\mu$		Intact Bacteria (%)
pH	Temperature (°C)	In 0.1M Sucrose	In Water (calc.)	
6.5	15	0.082	0.086	6
6.5	0	0.276	0.288	20
5.5	15	0.146	0.156	11
5.5	0	0.452	0.475	34

absorption measurements indicate that there is greater leakage of material absorbing at this wavelength at the higher temperature and pH. However, even after 6 days, none of the preparations show greater than 20 per cent. of the absorption observed on complete lysis. Since 260  $m\mu$  absorbing material is likely to be lost by chemical reaction and adsorption, quantitative deductions are unreliable.

Protoplasts show an increase in optical density at 500  $m\mu$  which is immediate at pH 5.5 and delayed at pH 6.5. Bacteria at pH 5.5 exhibit a trend similar to protoplasts at pH 6.5. Of particular interest are the bacteria stored at pH 6.5 and 15°C which exhibit a decrease in light scattering and approach the optical density reached by protoplasts stored under the same conditions. This observation suggested that at this stage the two preparations may be essentially identical and the bacteria spontaneously converted to protoplasts. In Table 1 the optical density at 500  $m\mu$  is given for each of the four aged bacterial suspensions diluted to 0.1M sucrose concentration which is sufficient to lyse any protoplasts which may have

formed. By allowing for the known effect of sucrose on the established curve calibrating optical density and bacterial concentration, the percentage of bacteria not lysed was calculated. These values, also shown in Table 1, indicate substantial conversions of bacteria to "protoplasts". It has been assumed that aged bacteria have similar light-scattering properties to fresh bacteria. Since the results in Figure 1 suggest that, at pH 5.5 at least, the scattering of light increases on aging, the bacteria calculated as surviving unchanged will tend to have been overestimated.

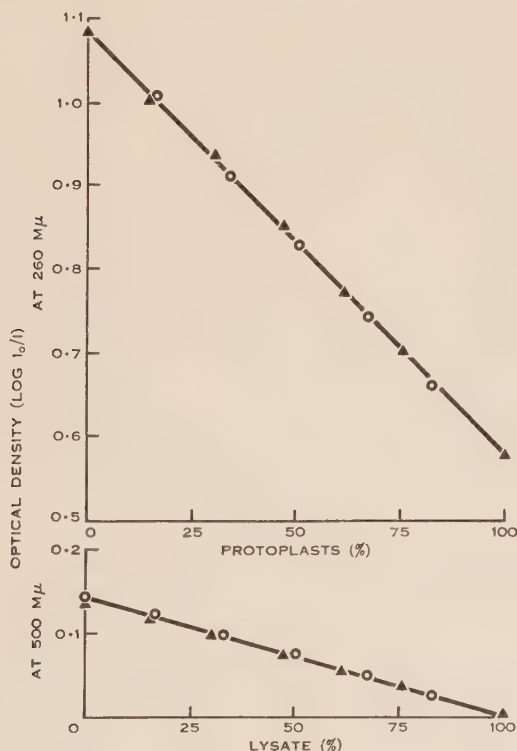


Fig. 2.—Optical properties of artificial mixtures of protoplasts and lysates of *M. lysodeikticus* in 1M sucrose plus 0.05M NaCl. Total concentrations 0.25 mg dry wt./ml. Two independent experiments indicated.

Viewed in the electron microscope, fresh protoplasts appear as discrete spheres, 0.7–0.8  $\mu$  in diameter. In this respect they differ from the bacteria from which they were derived, which invariably form clumps. On the other hand, aged bacteria believed to have changed spontaneously to "protoplasts" appear to be no longer aggregated. These latter preparations, however, no longer consist wholly of spheres, but show a very large proportion of dumb-bell shapes. Lysozyme-induced protoplasts which have aged in sucrose medium retain their spherical form and only occasional dumb-bell shapes have been observed.

(b) *Sensitivity of Protoplasts to pH*

The optical properties of artificial protoplasts-lysate mixtures are illustrated in Figure 2. Because of the linearity exhibited, the contributions to the optical density at  $260\text{ m}\mu$  due to specific absorption in protoplast suspensions and to non-specific scattering are additive. Thus the percentage of intact protoplasts can be calculated from the measurements at  $500\text{ m}\mu$  and the contribution to the measured optical density at  $260\text{ m}\mu$  due to these protoplasts may then be computed. The specific absorption at  $260\text{ m}\mu$  is obtained by difference.

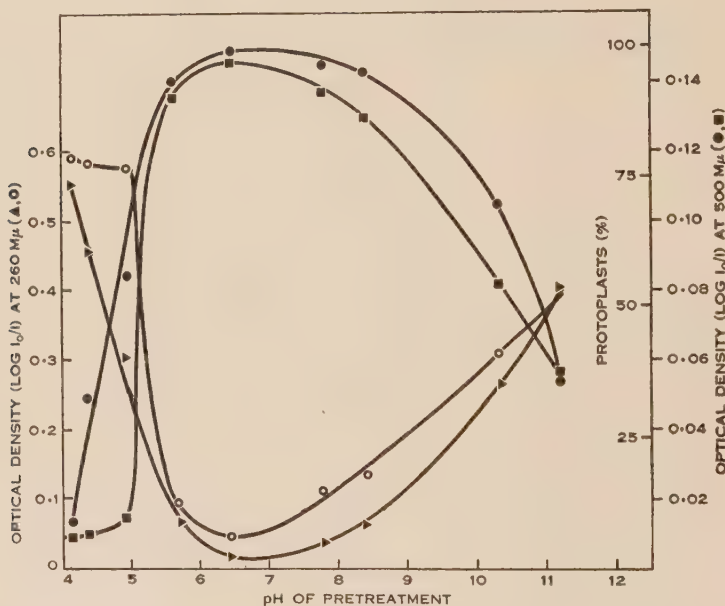


Fig. 3.—Stability to pH of protoplasts of *M. lysodeikticus*. Protoplast suspensions returned to pH 6.5 by 1/10 dilution with 1M sucrose. Conditions of pretreatment: protoplasts 2.5 mg/ml in 1M sucrose; 20°C. Incubation for 0.5 or 2.5 hr. Optical density measured at  $500\text{ m}\mu$ : ● 0.5 hr pretreatment; ■ 2.5 hr pretreatment. Optical density calculated for specific absorption at  $260\text{ m}\mu$ : ▲ 0.5 hr pretreatment; ○ 2.5 hr pretreatment.

The results of the optical density measurements at  $500\text{ m}\mu$  and the calculated specific absorption at  $260\text{ m}\mu$  are plotted against pH of treatment in Figure 3. A high degree of damage to the protoplasts is associated with a low optical density at  $500\text{ m}\mu$  and a high  $260\text{ m}\mu$  absorption. The action on the protoplast membrane is time dependent and incomplete in 30 min. A much sharper delineation of the range of stability is observed after 2.5 hr exposure. For this exposure the curves at  $500$  and  $260\text{ m}\mu$  both indicate that the onset of damage under acidic conditions occurs quite suddenly in a narrow range of pH slightly above 5. Over the range of pH 5.5–8.0 the protoplasts are relatively stable, but as the pH is raised they again become unstable. The onset of alkali damage is more gradual than that by acid.



## IV. DISCUSSION

During storage of bacteria and protoplasts, it is evident that considerable changes occur in the membrane systems of the organisms. However, the majority of protoplasts are not disrupted by osmotic explosion during storage, indicating that the leakage of 260 m $\mu$  absorbing compounds which occurs is not accompanied by breakdown in the impermeability to sucrose of the osmotic barrier. Nevertheless, the use of aged preparations for biophysical investigations appears inadvisable. This has been confirmed in other work (Gilby and Few 1957*a*, 1957*b*) in which the rate of lysis by detergents was observed to increase rapidly with protoplasts older than 8 hr. In view of the composition of the storage medium, it is not likely that growth of protoplasts would be supported. Indeed, although Jeynes (1957) has reported growth of protoplasts produced from bacteria by other methods, growth of lysozyme-induced protoplasts has not yet been observed even when nutrients are present. With aged protoplasts, however, some few dumb-bell shapes similar to those observed by McQuillen (1955*b*) have been noted here in electron-micrographs of formalin-fixed preparations. The two main factors believed to contribute to the observed increase in turbidity of protoplast suspensions are (1) shrinkage of protoplasts due to leakage of intracellular material, and (2) changes in the refractive index difference at the cell/medium interface altering the light-scattering properties.

With lysozyme rigorously excluded, the autolytic conversion of *M. lysodeikticus* to "protoplasts" can, under favourable conditions, be almost complete although apparently slower than with *Staphylococcus aureus* (Mitchell and Moyle 1957). Most of the *M. lysodeikticus* protoplasts resulting from aging are observed by electron-microscopy to exhibit a dumb-bell shape, the proportion being many times greater than in protoplasts aged after lysozyme treatment. It seems probable, therefore, that the steps towards division in the aging bacteria may have occurred before the attainment of the "protoplast" condition. In considering autolysis, Welsch (1958) has pointed out the difficulties in deciding between the presence of a truly bacteriolytic agent or an inducer of autolysis through secondary causes. The presence in the *M. lysodeikticus* cell sheath of an autolytic enzyme system cannot be excluded (Mitchell and Moyle 1956*b*). In the present experiments, the bacteria are grossly deprived of nutrient and do not divide to form the osmotically fragile naked cells observed by Meadow, Hoare, and Work (1957) and Rhuland (1957) when *Escherichia coli* is deprived only of a single agent believed essential to the synthesis of cell wall material. The fact that aged *M. lysodeikticus* bacteria are almost all dumb-bell shaped could mean, on the other hand, that the processes involved in the conversion to protoplasts are intimately linked with those of cell wall production and division. The latter processes may take place at a definite stage during growth, which here cannot proceed past a certain point. The observed loss of 260 m $\mu$  absorbing material indicates disorganization of the cytoplasmic membrane, a structure almost certainly involved in the formation of the cell wall. Whether the primary effect of interference with the cell nutrient supply during senescence is to inhibit the process of cell wall synthesis or to induce autolysis by breakdown of internal organization, it is therefore not possible to decide. Failure to observe cell wall fragments when osmotically sensitive, aged bacteria are lysed by dilution of the protective medium could favour the latter alternative.

Freshly prepared *M. lysodeikticus* protoplasts are stable in the range pH 5.5–8.0. Outside these limits, the trends shown by the measurements of optical density at 500  $m\mu$  and the calculated specific absorption at 260  $m\mu$  are closely parallel. If the optical density at 260  $m\mu$  is calculated only on the basis of protoplasts lysed as indicated by measurements at 500  $m\mu$ , agreement with the results in Figure 3 is within 5 per cent. This suggests that there is very little leakage of 260  $m\mu$  absorbing intracellular material without an accompanying loss of structural integrity and disruption of the protoplasts. Thus, rather than the initial action being to induce leakage of intracellular components, the primary breakdown of the protoplasts as a response to pH appears to be due to the destruction of the impermeability of the protoplast membrane to the stabilizing sucrose with consequent osmotic explosion. In previous work (Few, Fraser, and Gilby 1957) it has been shown that the onset of the leakage of 260  $m\mu$  absorbing material from intact *M. lysodeikticus* occurs at pH 4.4, which may be compared with the stability threshold of pH 5.5 for protoplasts. It is generally agreed that it is the protoplast membrane which maintains the osmotic function of bacteria (Mitchell and Moyle 1956*b*). With bacteria, the presence of the negatively charged cell wall will affect both the access of hydrogen and other ions to the protoplast membrane and the escape of intracellular constituents once this membrane is damaged. The observations support the view that the onset of damage to the protoplast membranes resulting in their becoming permeable to intracellular material occurs only under more drastic conditions than those sufficient to disrupt protoplasts by osmotic explosion. Furthermore, when *M. lysodeikticus* is titrated with hydrochloric acid, the barriers to the entry of hydrogen ions are only completely broken down at pH 3 (Gilby and Few 1958).

With the information at present available on the composition and structure of the membranes of *M. lysodeikticus* it is not possible to reach precise conclusions concerning the nature of the changes which produce the effects on cell permeability described. Chemical analysis (Gilby, Few, and McQuillen 1958) indicates the presence of protein, lipid, and polysaccharide material in the protoplast membrane. Unpublished work by Few, Gilby, and Seaman using micro-electrophoresis supports the "protein-lipid sandwich" theory as the basis of protoplast membrane structure, with the added possibility of the carbohydrate contributing to a triplex formation. It is unlikely that such a complex structure would respond in any simple way to changes in its environment.

#### V. ACKNOWLEDGMENTS

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# THE ONSET AND DURATION OF DIAPAUSE IN EGGS OF *ACHETA COMMODUS* (WALK.) (ORTHOPTERA)

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[Manuscript received July 9, 1959]

## Summary

The stage of morphogenesis at which diapause supervenes in eggs of the common field cricket *A. commodus* was critically determined, and found to be the 46-hr stage in terms of the series described by Brookes (1952). The examination revealed a difference in morphology between diapause and non-diapause embryos.

The onset of diapause is governed by temperature. Low temperatures induce diapause, whereas high temperatures tend to avert it. The change-over in response occurs at about 23°C below which all viable eggs enter diapause. Although exposure to 12·8°C for several weeks during pre-diapause enables development without delay at an incubation temperature of 26°C or higher, eggs held constantly at 12·8°C entered diapause.

The capacity of high temperature to avert diapause was used as a means of determining the time of exposure to a diapause-inducing temperature necessary to ensure onset of diapause in 90 per cent. of the viable embryos and also the stage of development of the embryo most sensitive to the effect of temperature. These experiments indicated that conditioning for diapause occurred several days before the actual onset, and that the maximum sensitivity was just prior to this stage.

The effect of constant temperature on the duration of diapause was measured at 23·3, 26·7, and 29·4°C. Diapause was terminated effectively at each temperature, with the minimum duration at 29·4°C. Preliminary exposure to 12·8°C for 21 days in the control treatment led to uniform, instead of extended, hatching.

A comparison of the effect of low temperature on pre-diapause and diapause eggs showed that those in diapause required considerably longer treatment for the elimination of diapause. From this, and other evidence, it is concluded that prevention of the onset of diapause by preliminary exposure to low-temperature treatment may not be the same process as termination of diapause, and that until more is known the term "diapause development" should be reserved for the latter.

## I. INTRODUCTION

Diapause is one means by which certain species of insects are able to survive in environments that are periodically unfavourable to their development. The subject is of interest to the economic entomologist in that a knowledge of its mechanism may enable it to be manipulated by artificial means and its protective value to the insect destroyed.

The variations of diapause and the conditions under which it occurs are innumerable. The immense amount of literature on the subject has been reviewed a number of times, more recently by Andrewartha (1952) and Lees (1955). Accordingly only references having specific application to the work in this paper will be mentioned here.

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There is now considerable evidence that embryonic and postembryonic diapause differ in several important respects, although this does not remove the possibility that the final cause of the arrest may be the same. Williams (1952) has shown that in the pupal diapause of the giant silkworm *Platysamia cecropia* (L.) the arrest of development is associated with an endocrine failure. The secretion of the growth and moulting hormone from the prothoracic gland is suppressed by the brain in response to external stimuli.

Such a mechanism cannot be the means by which development is brought to a halt in the embryo prior to the existence of the brain and endocrine system. Nevertheless, the endocrine system of the parent is involved. Fukuda (1952) has shown that in the silkworm *Bombyx mori* (L.) the brain controls the release of a hormone from the suboesophageal ganglion of the parent female. Hasegawa (1957) has isolated the active principle which, when injected into pupae of non-diapause stock, produced moths which laid diapause eggs. Morohoshi (1959) has shown that voltinism in *Bombyx* is determined by a balance between the hormones from the corpora allata and suboesophageal ganglion. In *Bombyx* and other examples of embryonic diapause, the embryo develops to a definite stage of morphogenesis before the arrest occurs. The critical stage varies with the species concerned (Lees 1955). The study of embryonic diapause has a distinct advantage in that once oviposition has occurred there is no complication by reactions from the brain. *Acheta commodus* (Walk.), the test insect in the studies described hereunder, has the additional advantage that the onset of its embryonic diapause can be affected by the environment of the egg.

In *Acheta* temperature is the important environmental factor controlling the onset and duration of diapause. Browning (1952*a*, 1952*b*) has studied certain aspects of the effect of temperature on the diapause of this insect, mainly in relation to the pre-diapause stages. He found that pre-diapause eggs exposed to low temperature for several weeks did not enter diapause when incubated at a temperature of 26°C or higher. This he ascribed to the completion of diapause development before the onset of diapause. The most favourable temperature for this purpose was approximately 13°C.

The present paper is concerned with the stage of morphogenesis at which diapause supervenes, and with the effect of different levels of temperature on both the onset and duration of diapause.

## II. MATERIALS AND METHODS

The field cricket has only one generation per annum in southern Victoria and egg laying extends for about six weeks during the autumn. Continuity of supplies of eggs for experimental work was maintained by rearing crickets in constant-temperature cabinets. This method was developed in 1953 in connection with laboratory insecticide tests against this pasture pest.

The immature stages were reared at about 30°C, in order to promote rapid growth, and transferred at maturity to a cabinet kept at 26.7°C for oviposition.

Comparisons were made between the diapause behaviour of eggs from crickets collected in the field and those reared in the laboratory, and no greater variation

was noted than between different batches of crickets from the field. As a further check the cultures were renewed annually by replacements from the field in order to avoid the possibility of selection. The temperature at which the culture is reared does not appear to affect the diapause behaviour of the eggs. A small proportion of eggs that did not develop was present in both the field and culture samples.

Eggs for experimental work were obtained by placing trays of moist sand in the cages for several hours during which time the eggs were laid singly at a depth of  $\frac{1}{2}$ – $\frac{3}{4}$  in. in the sand. The trays were then removed and the eggs sieved out under water. The swirling water during this procedure ensures a good mixture of eggs from the various females. The eggs were then tipped on to moist blotting-paper and counted into tubes. Large supplies of eggs can be obtained within a few hours by withholding the oviposition trays for several days beforehand. This does not affect the subsequent development of the eggs.

After a considerable number of tests of various containers for holding the eggs, air-tight plastic tubes 1 by 1 in. were found to be the best. At the bottom of each tube was placed a disk of blotting-paper, standard in size, weight, and moisture content. The tubes were left for 24 hr, and then examined for any signs of a change in the moistness of the paper. Any tube in which this occurred was replaced, on the assumption that it was not air-tight. After the eggs had been counted into the tubes, these were put into a double-walled plastic box having a layer of moistened plaster of paris across the base. This served the double purpose of maintaining high humidity in the box and provided additional heat capacity to damp out temperature fluctuations. These precautions were necessary in order to avoid variations in the moisture level from either evaporation or condensation. Such differences can affect the rate of development which, in turn, can alter the number of eggs hatching within the prescribed time.

The eggs were transferred into the tubes by means of a camel-hair brush and, as far as practicable, all were placed flat so as to make equal contact with the moist surface.

Comparisons between treatments were made on the basis of the percentage of eggs reaching the "eyespot" stage (Plate 1, Fig. 2(h)) in a time considered equivalent to that taken by eggs that develop without evidence of delay. These will be referred to as diapause-free. Eyespot counts were preferred to hatching counts as some cannibalism can occur after hatching. All stages at eyespot were included in the count, which was made as soon as the first eggs reached the most advanced eyespot stage. In this way an automatic correction was obtained for differences in the rate of development at each temperature, since the latter affects the number of days over which development can be considered diapause-free. After counting, the eggs were held until hatching was complete so that they could be examined for any evidence of abnormality attributable to particular treatments.

At the conclusion of the period of experimental observation, more detailed information on the stage of development reached by the embryo was obtained in certain of the tests. This was done by immersing the eggs in water and examining them under a microscope, by which means it was possible to record them as non-viable, pre-diapause, diapause, or the particular stage of post-diapause development.

## III. RESULTS

(a) *Stage of Embryonic Development at which Diapause Supervenes*

There is no record of any accurate determination of the stage of embryonic development at which diapause supervenes in the eggs of *Acheta*. Andrewartha (1952) included *Acheta* as an example of the group in which "onset occurs while the embryo is at an early stage, usually before segmentation is complete." Browning (1952a) suggests that it is about the fourth-day stage of development. Lees (1955), presumably quoting from Browning, gives the  $3\frac{1}{2}$ -day stage. In each case the stage of development referred to is that reached by the modal number of embryos when they are held for the time stated, at a temperature of  $25.2^{\circ}\text{C}$ , as described by Brookes (1952). These eggs were previously exposed to low temperature, and, therefore, not subject to diapause.

In this experiment, and those following, the stage of development of the embryo is referred to by a number that enables it to be identified in the series described by Brookes. Thus "stage 48" corresponds to the morphogenetic development listed by Brookes as having been reached after 48 hr at  $25.2^{\circ}\text{C}$ . The time unit has been omitted as it does not apply to other temperatures.

The morphogenesis of large numbers of embryos was traced with relative ease by direct examination of the embryo, after clearing the eggs by a method previously described (Hogan 1959). The technique for clearing is equally effective for diapause or non-diapause eggs but prior to water uptake by the eggs clearing is more rapid, and lower temperatures should be used.

The characteristics of the embryos on entry into diapause were observed at  $23.3$ ,  $26.7$ , and  $29.4^{\circ}\text{C}$ . A total of 150 eggs was held at each temperature and a sample of 20 eggs taken from each immediately before, during, and after the onset of diapause. Further samples were taken at intervals appropriate to the temperature. The timing of the sampling was based on preliminary examinations which showed that the onset of diapause occurred prior to water uptake. Supplementary observations on the comparative morphology of diapause and non-diapause embryos were made on a further series of eggs, after the main test had been concluded.

Considerable variation occurred in the rate of development of embryos at each temperature. A small proportion of the eggs showed no development after oviposition, presumably being infertile or non-viable. At each temperature the arrest of development took place at the stage shown in Plate 1, Figure 1, estimated to be about 2 hr younger than the 48-hr embryo illustrated by Brookes. It will be referred to as stage 46. After some time, depending on the temperature, the accumulation of embryos included stages 46–48 (Fig. 1). It was concluded, therefore, that diapause takes place at the stage 46, and that in the process of resuming normal development, the embryo gradually drifts through to stage 48. There was no indication of any further delay once the embryo had developed beyond stage 48.

Embryos in diapause were observed to be smaller and more compact than non-diapause embryos in the same stage of morphogenesis, the difference being greatest at the lower temperatures. In Plate 1, Figure 1, typical diapause embryos



are illustrated, while Plate 1, Figure 4, shows a diapause and non-diapause embryo. The proportion of larger embryos present at each temperature agrees with the expected proportion of non-diapause eggs (see Section III(b)), but the difference in size is less marked at the high temperatures.

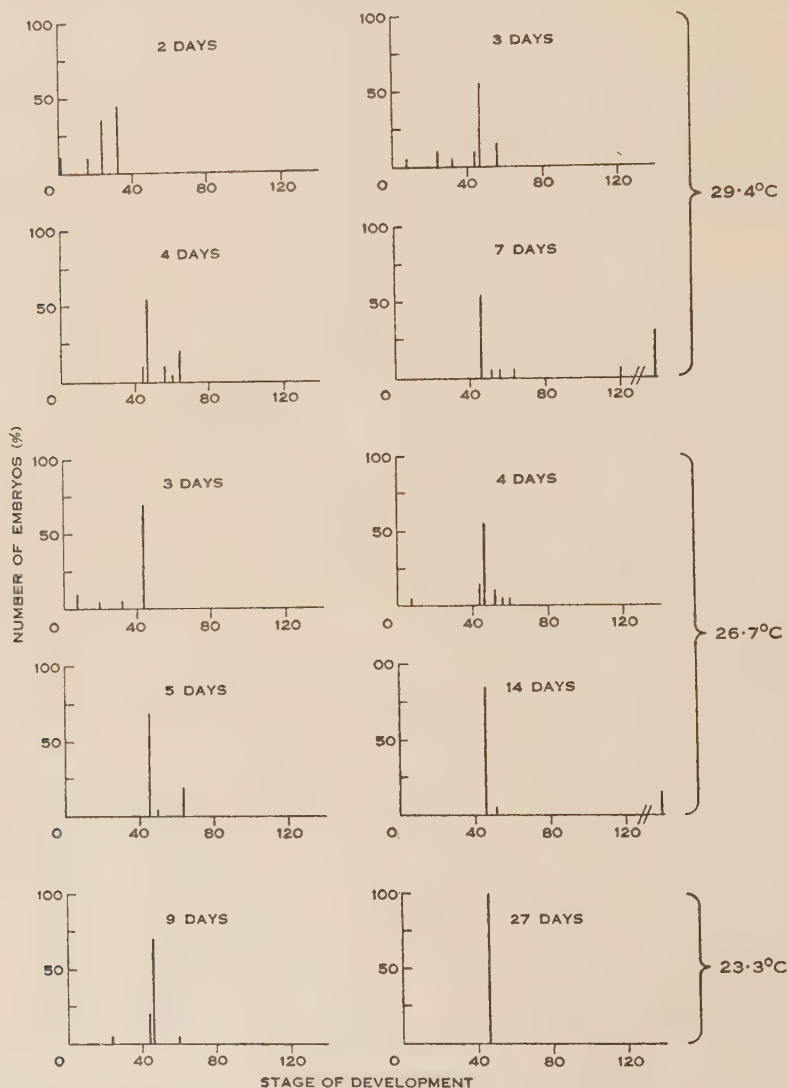


Fig. 1.—Stage of development reached by embryos after different periods at three temperatures. Note accumulation in stages 46–48.

In the course of development the eggs of *Acheta* absorb moisture almost equal to their own weight (Browning 1953). In diapause eggs, water uptake commences just as the embryo reaches the diapause stage, and is completed after the arrest of development takes place. In non-diapause embryos, on the other hand, develop-



ment continues during water uptake. After 4-4½ days (25.2°C scale) a clear space appears at the posterior end of the egg and this remains up to the end of the 6th day, after which the egg revolves. This clear space also appears in the diapause egg after a similar time, but the embryo is at a different stage of morphogenetic development, viz. stage 46 (Plate 1, Figs. 6 and 7).

(b) *Effect of Temperature on the Inception of Diapause*

It has been pointed out by Lees (1955) that as a rule high temperatures tend to avert diapause while low temperatures favour the "arrest of growth." However, the capacity of low temperature to remove diapause after onset is so well known that its importance in favouring the inception of diapause is apt to be overlooked. Andrewartha (1952) has remarked on this dual role.

TABLE 1  
PERCENTAGE OF EGGS HATCHING WITHOUT EVIDENCE OF DIAPAUSE WHEN  
INCUBATED AT THE TEMPERATURES INDICATED

Temperature (°C)	23.3	26.7	29.4	34.0	Control
Hatching (%)	0	16.0	64.0	83.3	91.3

Browning (1952*b*) noted that in *Acheta* as the incubation temperature was raised an increasing percentage of eggs developed without interruption. These observations have been confirmed and extended.

Six replicates of 25 eggs per treatment were held at temperatures of 23.3, 26.7, 29.4, and 34°C. Controls were held for 21 days at 12.8°C prior to incubation at 26.7°C. The other conditions of the experiment were as described in Section II.

From Table 1 it will be seen that there was no diapause-free hatching at 23.3°C, but that some prompt hatching occurred at all temperatures above this and that the percentage hatching increased as the temperature increased. The maximum increase was between 26.7 and 29.4°C when the hatching increased from 16 to 64 per cent. (Fig. 2). The form of the curve between these readings is based on subsidiary tests made after the main experiment. The non-diapause hatching at 34°C was 83.3 per cent. compared with 91.3 per cent. for the control. This difference was not statistically significant, whereas the difference between other treatments were highly significant.

Thus at high temperatures there is a strong tendency for diapause to be averted. This tendency diminishes with the lowering of temperature until at about 23°C it disappears. It would appear that 23.3°C is near the threshold of this effect, since an occasional egg develops without diapause at this level, whereas none have been recorded at the lower temperatures. The actual proportion of eggs from which diapause is averted by high temperature will depend on the original strength of diapause in the eggs. This, as has been pointed out by Browning (1952*b*), depends on factors, at present unknown, in the environment of the parent generation of crickets.

Browning also found that eggs held at low temperatures for several weeks shortly after oviposition and then incubated at 26°C, or higher, invariably developed without interruption. This would suggest that in *Acheta* eggs, contrary to the general rule,

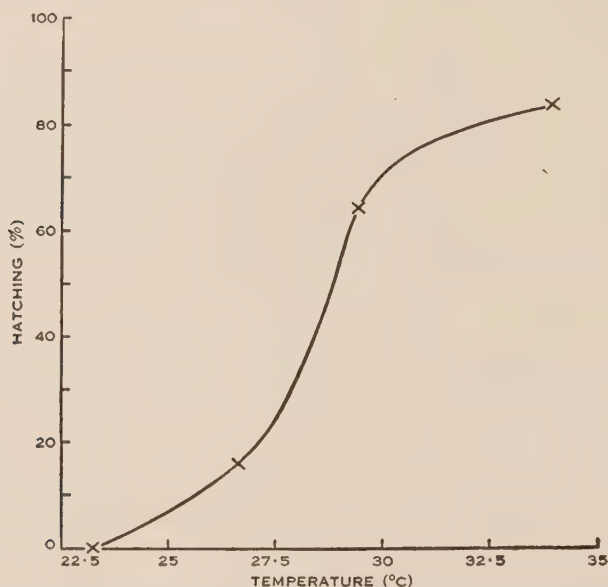


Fig. 2.—Effect of temperature on the induction of diapause. The proportion of eggs from which diapause is averted rises sharply at about 26°C.

diapause is averted by low temperatures. This, however, is not the case, as is shown by the following experiment in which a further series of eggs were held at 12.8, 14.8, 17.5, 21, and 29.4°C. All other conditions of the experiments were as in the first test.

TABLE 2  
PERCENTAGE OF NON-DIAPAUSE EGGS AT THE LOWER TEMPERATURES

Temperature (°C)	12.8	14.8	17.5	21.0	29.4
Non-diapause eggs (%)	0	0	0	0	75.0

The results of these experiments (Tables 1 and 2) show that at the lower temperatures all the eggs entered diapause. Observations on parallel samples of eggs, kept for that purpose and examined by the clearing method mentioned previously, showed that at 12.8°C development proceeded normally until the diapause stage was reached after which no further morphogenetic development took place. Whether they eventually resume post-diapause development or not, if held for a very prolonged period at 12.8°C, is not known. In further observations eggs which had entered diapause at 23.3°C and had been held at that temperature for

a further 3 months were cleared and examined, but morphogenesis had not been resumed.

The anomaly then arises that while low temperatures favour the onset of diapause, exposure to them during the pre-diapause stages accentuates the effect of high temperatures in promoting development without interruption. Browning (1952*b*) attributes this to the occurrence of "diapause development" during the preliminary exposure at low temperatures. This term was introduced by Andrewartha (1952) to describe the processes which occur during diapause and which must be completed before the embryo is competent to resume morphogenesis. Whether it is appropriate to apply the same term to processes which result in the complete elimination of diapause from the life history will be discussed later.

(c) *Sensitivity of Different Stages of Embryonic Development to a Diapause Temperature*

From Section III(b) it is clear that at a temperature of 23.3°C, or lower, diapause occurs in all eggs. It is not known, however, what period of exposure to such a temperature is required to cause the onset of diapause. Nor is it known whether conditioning for diapause occurs at a particular stage of pre-diapause development, over a number of stages, or in the latter event, whether some stages show greater sensitivity than others. One reason for investigating such influences is to enable proper standardization of the experiments on other aspects of diapause. If, for instance, the conditions existing over the first 24 hr after oviposition affect diapause behaviour, then this should be known and taken into account in the design of the experiments.

In other species of insects a considerable amount of information is available on the exact stage of the life cycle at which diapause supervenes, but there is relatively little on the stage at which conditioning for diapause occurs. Way and Hopkins (1950) investigated the larval diapause of *Diataraxia oleracea* (L.) to determine the stage at which photoperiod was operative in inducing diapause. Masaki (1957) found that the proportion of larvae of *Barathra brassicae* (L.) entering diapause was proportional to the length of the exposure to a diapause-inducing temperature. Other examples of larval and pupal responses are quoted by Lees (1955).

In *Acheta* the ability of high temperature to avert diapause in a high proportion of the eggs provides a means of measuring the requirements of the embryo for diapause induction and the responsiveness of the various pre-diapause stages of the embryo to the temperature of the environment.

In the following experiments the eggs were held at a temperature of 29.4°C, which averts diapause in a high proportion of the eggs, and a diapause-inducing temperature of 23.3°C substituted at the stages and intervals indicated in Figure 3. The interchange of temperatures was carried out over a period equivalent to 8 days at 23.3°C from the time of oviposition. This time was sufficient to ensure that all viable eggs would reach the diapause stage.\* This period was divided into eight

\* At 23.3°C the 46-hr stage in the terminology of Brookes is reached only after about 6 days incubation. This discrepancy is due partly to the lower temperature and partly to the fact that 2-3 days incubation at 25.2°C are required before the embryo reaches the "0 hour" stage described by Brookes (1952).

stages, each corresponding to one day's development at this temperature. Since the rate of development at  $29.4^{\circ}\text{C}$  is twice that at  $23.3^{\circ}\text{C}$  (Hogan, unpublished data 1958), it is a simple matter to substitute one temperature for another so that the same amount of embryonic development is completed at each temperature. One day at  $29.4^{\circ}\text{C}$ , for example, will enable the same amount of development as 2 days at  $23.3^{\circ}\text{C}$ . By holding a control group of eggs at a constant temperature of  $29.4^{\circ}\text{C}$  the effect of exposure to  $23.3^{\circ}\text{C}$  at different periods during pre-diapause development could be measured.

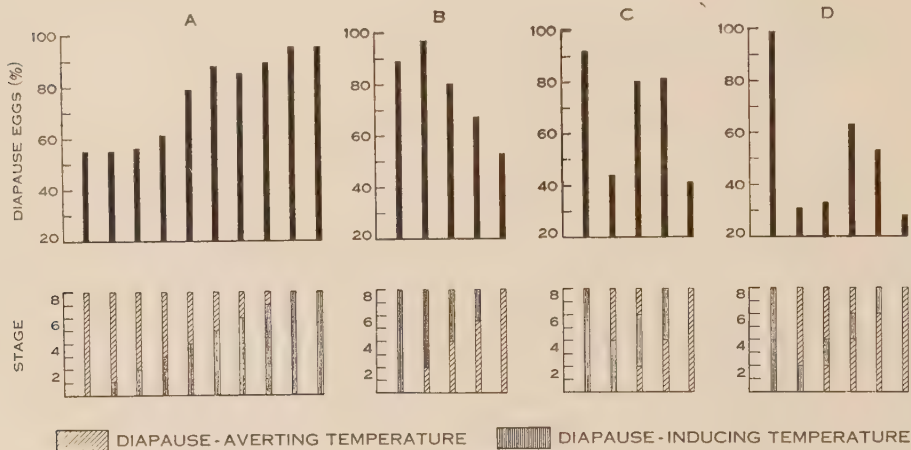


Fig. 3.—Diapause induction in pre-diapause embryos subjected to various temperature regimes as indicated.

In the first test, cumulative one-day periods at the diapause-inducing temperature,  $23.3^{\circ}\text{C}$ , were added from the time of oviposition up to a maximum of 8 days. Figure 3A shows that no effect was obtained until the third day, after which there was a rapid rise in the proportion of eggs entering diapause. This continued till the fifth day and was only slightly higher by the eighth day.

The lack of response over the first 2 days could be due to either insensitivity at this stage of development, or a too short exposure. The treatments were, therefore, reversed so that the shorter periods of exposure to the diapause-inducing temperature took place just prior to the diapause stage (Fig. 3B). Increments equivalent to 2 days at  $23.3^{\circ}\text{C}$  were used so that there were then only four subdivisions. The responses obtained show some effect from the 2 days treatment just prior to diapause, but to achieve the maximum effect a period of 6 days was necessary.

Both aspects were investigated by two further tests. These were carried out by interposing four successive 2-day intervals at  $23.3^{\circ}\text{C}$  in one case, and three 4-day intervals in the other. Figure 3D shows that for the 2-day exposures the fifth- and sixth-day interval was the most responsive, but that evidently the total time of exposure was too short to induce diapause in all eggs. It is apparent again from this test that this time of exposure has little influence when applied to the early stages of embryonic development.



The 4-day interval tests (Fig. 3C) indicated that this period of time was highly effective in inducing diapause, particularly when applied so as to include the fifth and sixth days. When these stages were included, approximately 90 per cent. of the full effect was obtained.

Thus over the first 2 days after oviposition the embryos are relatively insensitive to the diapause temperature except insofar as this may add to the effect of treatment applied later. The fifth and sixth days are the most responsive, but all stages after the first 2 days respond appreciably. In Section III(a) it was found that in the majority of the eggs the morphology of the embryo changes about the sixth day at 23.3°C to show the characteristics of either a diapause or a non-diapause embryo. Hence the most sensitive stage is judged to be just prior to stage 46, probably between stages 40 and 44. The response obtained on the seventh and eighth days can be accounted for partly by the variation in the rate of development, since some of the embryos would still be in the stage corresponding to the fifth or sixth day.

These experiments indicate that all but the earliest stages of pre-diapause development give some response to a diapause-inducing temperature, and that there is a period of maximum sensitivity near the fifth and sixth days of development under the conditions described, during which the embryo may or may not enter diapause according to environmental conditions at the time. To cause diapause in 90 per cent. of the viable eggs a minimum period of exposure of slightly more than half the pre-diapause development time is required, and this should coincide with the most sensitive stages, viz. that reached after 5-6 days after oviposition when held at a temperature of 23.3°C.

(d) *Effect of Low Temperature on Pre-diapause and Diapause Eggs*

It is known (Browning 1952a) that if eggs of *Acheta* are held at 13°C for several weeks and then transferred to a suitable incubation temperature, they will continue development without evidence of diapause. Browning refers to this as completion of diapause development. The completion of diapause development in this way would be most unusual. The only other comparable case appears to be *Melanoplus bivittatus* (Say). In this insect, eggs which do not reach the diapause stage by winter-time continue development without diapause in the following spring. This is referred to simply as "diapause averted" by Church and Salt (1952).

In the present series of experiments it has been shown (Section III(b)) that eggs held at 13°C enter diapause and are still in this condition after a further 3 months. This would suggest that low temperature does not terminate diapause and also provides clear evidence that the low-temperature treatment of pre-diapause eggs does not eliminate diapause. However, the data on this subject can be interpreted to mean that diapause development does proceed, since the reduction in the proportion of eggs entering diapause after exposure to low temperature for a limited period (15-30 days) is most readily explained on the assumption that diapause development has proceeded, but has not been completed.

Further evidence has been sought by comparing the responses from the pre-diapause eggs with those from diapause eggs given the same low-temperature treatment.

For this purpose 1200 eggs, up to 16 hr old, were divided into two groups, one of which was transferred immediately to low temperature, and the other to 23.3°C for 14 days in order to induce diapause. Each group was subdivided into three lots of six tubes and held for 30, 45, and 60 days at 12.8°C followed by incubation of one-half of the tubes at 23.3°C and the other half at 26.7°C. Controls had no preliminary low temperature treatment.

There was a marked difference between the pre-diapause and diapause eggs with respect to the period of low temperature required to remove, or prevent, diapause (Table 3). The effect was masked at an incubation temperature of 26.7°C because the hatching at this temperature was high for all treatments. It is clear from the hatching at 23.3°C, however, that once diapause supervenes, the length of exposure to low temperature necessary to eliminate diapause was at least twice that required to prevent its inception. Of the treatments for 30, 45, and 60 days at low temperature, only the 60-day treatment of the diapause eggs gave hatching comparable to that obtained after 30 days at low temperature of the pre-diapause eggs.

TABLE 3

PERCENTAGE OF EGGS HATCHING FREE OF DIAPAUSE FOLLOWING THE TEMPERATURE REGIMES APPLIED TO PRE-DIAPAUSE AND DIAPAUSE EGGS

Time at 12.8°C (days)	Control		30		45		60	
Incubation temperature (°C)	23.3	26.7	23.3	26.7	23.3	26.7	23.3	26.7
Pre-diapause eggs (%)	—	28	88	84	84	88	85	92
Diapause eggs (%)	4	8	16	80	56	92	80	92

The difference is even more marked when it is considered that after 30 days at low temperature only 16 per cent. of the diapause eggs resumed development, while 88 per cent. of the pre-diapause eggs developed without evidence of delay.

(e) *Effect of Constant Temperature on the Elimination of Diapause*

In the majority of the insect species investigated the elimination of diapause, when it occurs, is governed wholly, or in part, by temperature. The striking feature of the temperature requirements, both for different species and for embryonic or post-embryonic stages, is their comparative uniformity. Andrewartha (1952) has observed that, with few exceptions, completion of diapause development takes place most efficiently in the temperature range between 0°C and the threshold of development of the insect.

*Acheta* differs from most other insects in that diapause development can proceed at normal developmental temperatures. Browning (1952a) showed that eggs held at 26.7°C will eventually emerge from diapause. The optimum temperature for elimination of diapause from eggs in diapause was not determined, but he found c. 13°C to be the most efficient temperature for the treatment of pre-diapause eggs.

Since temperatures in the region of  $30^{\circ}\text{C}$  are considerably more effective than  $26.7^{\circ}\text{C}$  in averting diapause, it was decided to compare the effectiveness of  $29.4^{\circ}\text{C}$  with that of moderate and low temperatures in eliminating diapause.

There is a possibility that the intensity of diapause may be affected by the temperature at which onset takes place. Hence, if the eggs are held at a constant temperature from the time of oviposition to hatching, a comparison of the effectiveness of each temperature in eliminating diapause will not be valid, since the strength of diapause may have been affected by the inception temperature.

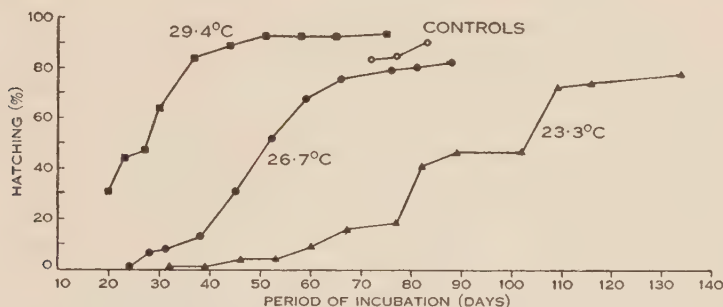


Fig. 4.—Cumulative frequency of eggs hatching at different constant temperatures after being induced to enter diapause at  $23.3^{\circ}\text{C}$ . The controls were kept at  $12.8^{\circ}\text{C}$  for 21 days and were then incubated at  $26.7^{\circ}\text{C}$ .

In this experiment, therefore, diapause was induced in the eggs by holding them for 10 days at  $23.3^{\circ}\text{C}$ . These were then divided into four treatments, viz. incubation at  $12.8$ ,  $23.3$ ,  $26.7$ , and  $29.4^{\circ}\text{C}$ . The control treatment was held for 21 days at  $12.8^{\circ}\text{C}$  and was incubated at  $26.7^{\circ}\text{C}$ . Observations were made on the eggs at intervals governed by their rate of development. The counts for comparison of treatments were made at the eyespot stages, but all eggs were held till hatching, to enable detection of any abnormalities which might occur from the effect of high temperature on diapause eggs.

Development was resumed and hatching completed at all temperatures except  $12.8^{\circ}\text{C}$  in the times shown in Figure 4. At each temperature the time taken to complete development varied considerably between individual eggs. At  $23.3^{\circ}\text{C}$  it ranged from c. 30 to 129 days; 50 per cent. had hatched after 86 days and the peak of emergence was from 77 to 104 days, during which 55 per cent. of the eggs hatched. The 50 per cent. level appears to be the most suitable parameter for the comparison between treatments.

At the higher temperatures the completion of morphogenesis was accelerated so that at  $26.7^{\circ}\text{C}$  50 per cent. of the diapause eggs hatched after 46 days, and at  $29.4^{\circ}\text{C}$  after 26 days. At  $29.4^{\circ}\text{C}$  the percentage hatching, 94.7 per cent., was equal to that from the control treatment, i.e. with preliminary low temperature. No abnormalities in hatching were observed.

It can be said, therefore, that the termination of diapause is efficient at high temperature. Moderate temperatures are less effective.



Low-temperature treatment is in a separate category. At a constant temperature of  $12.8^{\circ}\text{C}$  eggs developed until they reached the diapause stage. Morphogenesis then ceased. It was not resumed during the 3 months that these eggs were held after entering diapause.\*

Thus, diapause was terminated at all temperatures above  $12.8^{\circ}\text{C}$ , but most readily at the higher temperatures. To obtain uniform hatching in the minimum time, the double treatment consisting of a preliminary low-temperature treatment followed by incubation at a high temperature is the most effective.

#### IV. DISCUSSION

From the foregoing experiments it is clear that the temperature of the environment affects the diapause behaviour of *A. commodus* eggs in a number of interacting ways. The tendency to enter diapause, the intensity with which it is induced, and the time taken for its elimination, all vary with the temperature regime. The reactions also suggest that while *A. commodus* is well adapted to the environment of southern Victoria, it may also possess a capacity to develop a multi-voltine life cycle in a warmer climate.

Under the conditions normally prevailing in southern Victoria the life cycle is uni-voltine. Since the eggs are laid in the soil at a depth of about  $\frac{3}{4}$  in., and the pasture growth adds further protection from the effects of direct sunlight, the temperatures they experience are more uniform than the air temperature and approximate to the mean air temperature. Early in autumn the mean air temperature is about  $19^{\circ}\text{C}$  and gradually declines to about  $14^{\circ}\text{C}$ . These temperatures are within the range at which all eggs enter diapause (Section III(b)) and, therefore, diapause would be expected to be obligate under field conditions. Samples of eggs, totalling some hundreds, taken from the field during June and cleared for examination of the embryo have confirmed these expectations (Hogan, unpublished data 1958).

As the temperatures decline with the approach of winter, the diapause is weakened. Thus, the stronger diapause during the warmer weather of early autumn means that the eggs laid at this time are less likely to complete diapause development and hatch out at the wrong time.

The function of diapause under Victorian conditions appears to be not only to ensure overwintering in the egg stage, but also the prevention of hatching during the spring. Wet, cool conditions, such as normally occur at intervals in spring, appears, from laboratory observations, to be inimical to development. Diapause causes hatching to be delayed until early summer when high temperatures and drier conditions normally occur.

The reactions of *Acheta* eggs to high temperatures appear to give some support to the ideas of Salt (1947) in relation to larvae of *Cephus cinctus* (Nort) that both low and high temperatures are required for the complete elimination of diapause. *Acheta* shows a difference from *Cephus* in that high temperature alone is quite effective in terminating diapause. However, when both low and high temperatures are used,

\* In more recent work, diapause eggs held at  $14.4^{\circ}\text{C}$  commenced to emerge from diapause after about 100 days.

the higher the temperature at which they are incubated the shorter the time they need at low temperature to enable morphogenesis to be resumed. This suggests that the transfer to high temperature provides a stimulus which overcomes the residual diapause in the egg, or, alternatively, that high temperature may be an actual requirement for the complete elimination of diapause, as suggested by Salt.

Further evidence on this would be provided by the length of time taken for the embryo to resume morphogenesis after transfer to high temperature. Data on this is being obtained.

There have been indications in the course of the foregoing investigation that the onset of diapause can be affected by moisture conditions, but so far it has not been possible to obtain experimental proof of this. Moisture has been shown to be a factor in the diapause of *Melanoplus differentialis* (Thos.) by Bucklin (1953) and by Slifer (1958), but in *Melanoplus* it is the entry of moisture into the egg via the hydropyles that controls diapause. In *A. commodus* water enters both the diapause and non-diapause egg at approximately the same time after oviposition. It is the availability of moisture to the embryo that is in question.

Lees (1952) excludes *Acheta* from those species in which hydration of the egg might be "insufficient to permit active embryonic growth", and includes it as a species in which "the active control of diapause must be exercised through some agency other than water." He bases this on a statement, attributed to Browning, that the egg enters diapause in a fully hydrated condition. It is now known that diapause ensues before hydration of the egg, so that this reasoning no longer applies.

The other reason that Lees gives for dissociating diapause and the moisture content of the egg in this insect is that, if the freshly laid eggs are chilled for an adequate period, "diapause development" is completed before the embryo has grown to the morphological stage. "All the water required for healthy post-diapause growth . . . is taken in subsequently".

There is reason to doubt the validity of this evidence also. Browning (1952a) used the term "completion of diapause development" to describe the prevention of onset of diapause in eggs that were given appropriate low-temperature treatment shortly after oviposition, and were then incubated at a higher temperature.

Whatever processes may lead to the termination of diapause can reasonably be described as "diapause development", and in this paper the term has been used in this sense.

However, there is no clear evidence that the failure to enter diapause after treatment of the pre-diapause eggs results from the same processes that cause the termination of diapause, even although low temperature is effective in each case. For one thing, it has been shown (Section III(d)) that it takes considerably longer to terminate diapause than it does to prevent the onset of diapause.

Moreover, the physical differences between diapause and non-diapause eggs must result from reactions set in train at an earlier stage of embryonic development. The failure to trigger these reactions is not the same as their removal after onset, even although the destruction of a diapausing agent may be involved in each case.

Until more is known of the processes that affect the onset of diapause and those that terminate diapause, it would seem undesirable to make the assumption that they are exactly the same by the use of the term "diapause development" to cover both phenomena.

Since the intensity of diapause seems to be affected by the temperature at which the onset of diapause takes place, the question of the factors affecting strength of diapause are of special interest and are being investigated.

#### V. ACKNOWLEDGMENTS

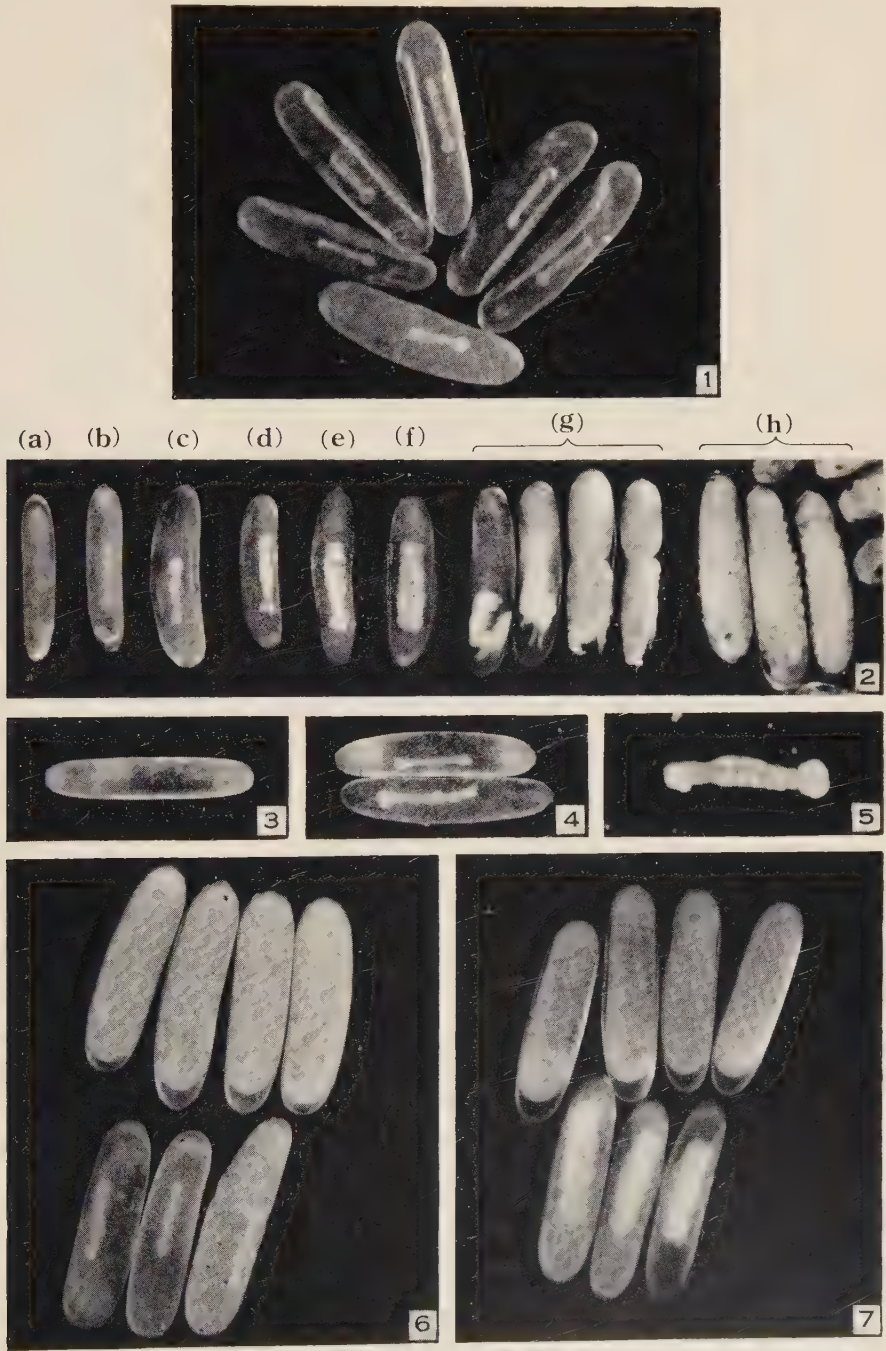
The author is indebted to Miss J. Kenyon for her able assistance in carrying out the tests, to Mr. R. Jardine, for the statistical aspects of the work, to Mr. M. P. Gellert for the preparation of the figures, and to Dr. F. H. Drummond for constructive criticism in the preparation of the manuscript.

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ONSET AND DURATION OF DIAPAUSE IN ACHETA COMMODUS





## EXPLANATION OF PLATE 1

Fig. 1.—Diapause embryos.

Fig. 2.—Stages of development of the embryo. In terms of Brookes' series they represent (a) 8 hr; (b) 24 hr; (c) 46 hr; (d) 56 hr; (e) 64 hr; (f)  $4\frac{1}{2}$  days; (g) rotation of embryo; (h) "eyespot".

Fig. 3.—Frontal view of the 24-hr stage.  $\times 9$ .

Fig. 4.—Diapause (upper) and non-diapause (lower) embryo at the same stage of morphogenesis.  $\times 9$ .

Fig. 5.—An embryo (48 hr) dissected from the egg to show details of its structure.  $\times 18$ .

Figs. 6 and 7.—Diapause (Fig. 6) and non-diapause (Fig. 7) eggs after the same period of incubation. The clear space at the posterior end of the eggs has developed to the same extent but when cleared (lower parts of Figures 6 and 7) the stage of morphogenesis is very different: stage 48 (Fig. 6), stage 108 (Fig. 7).



# NATURAL SELECTION FOR AN INTERMEDIATE OPTIMUM

By B. D. H. LATTER\*

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## *Summary*

Natural selection against extreme metric deviation is a process which is known to lead to the elimination of genetic variability in the particular quantitative character concerned. Recurrent mutation at loci affecting the character will be expected to oppose this tendency to genetic fixation, and the resulting equilibrium situation is discussed in detail in this paper.

The equilibrium genetic variance due to a given locus is shown to be a function only of the mutation rate at that locus, being independent of the magnitude of the average effect of the gene concerned. From the analysis it appears that recurrent mutation, in the face of natural selection favouring phenotypic intermediates, can account for only a small proportion of the genetic variance commonly found in natural populations, underlining the probable importance of heterozygote superiority in fitness as a mechanism conserving genetic variability.

The behaviour of the equilibrium population under artificial selection for extreme expression of the trait is discussed: the consequences of the model are found to be quantitatively similar to those expected on the basis of Lerner's model of genetic homeostasis.

## I. INTRODUCTION

Of the many problems confronting the student of evolution, one of the most interesting to those concerned with plant and animal improvement is the persistence of additive genetic variation for individual quantitative characters in natural populations. Abundant evidence of the existence of such genetic variation has been accumulated for an array of species, and it is the rule rather than the exception that artificial selection is able to bring about pronounced changes in the means of individual characters over only a small number of generations. Haldane (1954*a*) has discussed the factors known to be responsible for the retention of genetic variation in natural populations, including spatial and temporal clines, heterosis at individual loci, and the conflict between mutation and selection. The relative importance of these factors in maintaining variation for specific quantitative characters is, however, only very imperfectly understood.

It is clear that the concept of adaptive significance cannot be applied to isolated quantitative characteristics other than tentatively, for we must always bear in mind that it is the phenotype as an integrated whole which is the subject of natural selection. Nevertheless, we must suppose that for each metric character there exists an optimum value, related to the prevailing genotypic and external environment, and that the mean of a population under natural selection will have moved to approximate to this optimum by the time genetic equilibrium is reached.

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Fisher (1930) has shown that the action of natural selection in discriminating against those individuals which deviate from the optimum in the particular character concerned is such as to lead to the elimination of genetic variability rather than its preservation, and Robertson (1956) has contrasted this situation with that in which extreme deviants are unfit because of their greater degree of homozygosity than individuals approximating to the population mean (Lerner 1954). Robertson has shown the latter model to lead to useful predictions for artificial selection imposed on the population: "It was found that several different phenomena could be interrelated on this model—the relationship of fitness to deviation both in the equilibrium population and after artificial selection, the decline in fitness after such selection, and the rate of return to the equilibrium position when selection was suspended."

It is the purpose of this paper to consider in more detail the model of selection against metric deviation, with a view to setting out the expected consequences of the model under the influence of artificial selection.

## II. NATURE OF THE GENETIC EQUILIBRIUM

Let us suppose, following Haldane (1954*b*), that the decline in reproductive fitness with deviation  $x$  from the population mean of a given metric character is a continuous function of the form

$$1 - \exp(-\frac{1}{2}x^2/\sigma_f^2).$$

The scale constant  $\sigma_f^2$  is obviously related to the intensity of natural selection for the optimum value, being small if selection permits only those individuals close to the mean to reproduce, and large if a greater degree of latitude is tolerated. One of the most pertinent comparisons one would wish to make in a situation to which this model applied is that of the relative magnitude of  $\sigma_f^2$  and  $\sigma_p^2$ , the total phenotypic variance shown by the character under study. Haldane has in fact defined the intensity of selection for a normally distributed character to be the natural logarithm of the ratio of the phenotypic standard deviation prior to selection to that after selection, which comes out to be

$$I = \frac{1}{2} \log_e[1 + (\sigma_p^2/\sigma_f^2)], \quad \dots\dots\dots(1)$$

which equals  $\frac{1}{2}\sigma_p^2/\sigma_f^2$  for low intensities of selection.

Robertson (1956) has set out in full the derivation of an expression for the change in gene frequency per generation at an individual locus with additive effects on a normally distributed metric character, other effects of the locus being assumed to have no appreciable influence on reproductive fitness. If the difference in average metric value between the two alternative homozygotes at the locus is denoted by  $a$ , the change in gene frequency per generation under natural selection is given by

$$\delta q = a^2 pq(q-p)/8\sigma^2,$$

where  $\sigma^2 = \sigma_p^2 + \sigma_f^2$ , and  $p = 1 - q$ . The sign of  $\delta q$  depends only on that of  $(q-p)$ ;

if  $q$  is less than  $p$ ,  $\delta q$  will be negative and the value of  $q$  reduced each generation to an extent depending on the value of  $a$ , measuring the effect of this locus on the metric character. If  $q$  is greater than  $p$ , the effect of natural selection for the intermediate optimum will further increase  $q$  each generation. Gene frequencies in the population will therefore tend to zero or unity.

Opposing this tendency to genetic fixation there will only be recurrent mutation at loci affecting the character, and in a natural population we can expect the two sets of opposing forces to have resulted in an array of stable gene frequencies. Assuming forward and back mutation rates at the given locus to be of the same order, we may express the equilibrium situation algebraically by the relation

$$a^2 pq(q-p)/8\sigma^2 = \mu(q-p), \dots\dots\dots(2)$$

where the rate of mutation is denoted by  $\mu$ . It is easily shown that the genetic variance due to this locus is  $\frac{1}{2}a^2pq$ , which at equilibrium is equal to  $4\mu\sigma^2$ . Inequality of the forward and back mutation rates at the locus will have little effect on this deduction, provided  $\mu$  is taken to refer to the mutation rate from the more frequent to the less frequent allele. It is important to stress that, under the assumptions stated, the equilibrium genetic variance contributed by a single locus is a function only of mutation rate, and is independent of the magnitude of the average effect of the gene concerned.

### III. GENETIC VARIANCE DUE TO RECURRENT MUTATION

Let us now consider whether genetic equilibrium due solely to the conflict between mutation pressures and natural selection against phenotypic deviants can account for the amount of genetic variability commonly encountered in natural populations. For a character showing only additive genetic variation, the heritability ( $h^2$ ) is defined to be the ratio of the total genetic variance to the phenotypic variance in the population concerned, and it is not uncommon to find heritability values as high as  $\frac{1}{2}$ .

According to the model we are considering, the total genetic variance at equilibrium is  $4N\bar{\mu}\sigma^2$ , where  $N$  is the number of loci affecting the metric trait, and  $\bar{\mu}$  is the mean mutation rate at these loci. The equilibrium heritability is therefore given by

$$h^2 = 4N\bar{\mu}[1 + (\sigma_f^2/\sigma_p^2)].$$

We have no experimental evidence to indicate a likely value for the mean rate of mutation at individual loci affecting quantitative characters, but work with major genes in higher organisms would lead us to suggest a value for  $\bar{\mu}$  of approximately  $10^{-5}$ .

In terms of the intensity of natural selection ( $I$ ) the equilibrium heritability can be written (from (1))

$$\begin{aligned} h^2 &= 4N\bar{\mu}[1 + (1/2I)] \\ &= 2N\bar{\mu}/I \end{aligned}$$

approximately, provided the intensity of natural selection is low. To explain a



heritability value of  $\frac{1}{2}$  for a quantitative character we should therefore have to postulate an intensity of natural selection as low as say 1 per cent., with 250 genes segregating for the character. In view of the fact that the contribution of each locus to the total genetic variance is independent of the magnitude of its effect on the measured character, we might for some characters (e.g. body size) accept this number of genes as a possible figure. In general, however, it seems unlikely that the mechanism we are considering could of itself be responsible for the maintenance of the genetic variability shown by characters of high heritability.

#### IV. EFFECTS OF ARTIFICIAL SELECTION AND RELAXATION

Under individual selection for extreme expression of an additive genetic character, the change in gene frequency per generation at a given locus is expected to be

$$\delta q = (\bar{a}/2\sigma_p)pq,$$

where  $\bar{a}$  measures the intensity of extreme selection (Haldane 1930). At equilibrium, according to the model we are considering, the value of  $pq$  is  $8\mu\sigma^2/a^2$  from (2), so that the change in gene frequency per generation in the early stages of selection is expected to be

$$\delta q = 4\bar{a}\mu\sigma^2/a\sigma_p.$$

The change in gene frequency is therefore least for those loci with large effect on the character, and vice versa. However, the change in mean per generation due to a single locus is given by  $a\delta q$ , which is independent of  $a$ , so that the contribution of each locus to the advance in the mean under artificial selection is dependent only on the rate of mutation at that locus.

The decline in fitness with selection is readily predicted on the basis of the model we have set up: after a shift in the population mean of  $x_0$ , the mean fitness of the selected population in its natural environment is given by

$$\begin{aligned} & \frac{1}{\sqrt{2\pi}\sigma_p^2} \int_{-\infty}^{+\infty} \exp\left[-\frac{1}{2}\left(\frac{x-x_0}{\sigma_p}\right)^2\right] \exp\left[-\frac{1}{2}\frac{x^2}{\sigma_f^2}\right] dx \\ &= (\sigma_f/\sigma) \exp[-\frac{1}{2}x_0^2/\sigma^2]. \end{aligned}$$

The equilibrium population ( $x_0 = 0$ ) has average fitness  $\sigma_f/\sigma$ , so that the fitness of the selected population relative to that of the equilibrium population is

$$\exp[-\frac{1}{2}x_0^2/\sigma^2]. \quad \dots\dots\dots (3)$$

We can also predict accurately the behaviour of the selected population on the relaxation of artificial selection. Let us suppose that at a particular locus the equilibrium gene frequencies  $p, q$  have been changed to  $p^*, q^*$  corresponding to an overall change in the population mean of  $x_0$ . The average fitness values of the subpopulations corresponding to the three alternative genotypes  $A_1A_1, A_1A_2, A_2A_2$  are, from (3),

$$\exp(-\frac{1}{2}[(x_0 - ap^*)/\sigma]^2) : \exp(-\frac{1}{2}[(x_0 + a(q^* - \frac{1}{2}))/\sigma]^2) : \exp(-\frac{1}{2}[(x_0 + aq^*)/\sigma]^2),$$

where  $q$  is the frequency of the  $A_1$  allele. To a first approximation these fitness values are as  $1:1-s:1-2s$ , where  $s = ax_0/2\sigma^2$ . Hence the change in gene frequency under relaxation of selection is expected to be, per generation,

$$\delta q^* = \frac{ax_0}{2\sigma^2} p^* q^*.$$

The effect of natural selection on the relaxed population can be compared with that of artificial back selection of intensity  $i$ , which would lead to a change in gene frequency of  $(ia/2\sigma_p)p^*q^*$ , assuming  $\sigma_p^2$  to have been little altered in the course of selection. The effects of natural selection can then be seen to be equivalent to artificial back selection of intensity  $x_0\sigma_p/\sigma^2$ . Assuming the overall heritability of the character to have remained unchanged over the original period of artificial selection (a reasonable assumption in the light of experimental evidence for periods of up to 10 generations of moderately intense selection), the actual change in the mean of the selected population on relaxation in the first generation is given by

$$ih^2\sigma_p = h^2x_0\sigma_p^2/\sigma^2$$

towards the mean of the unselected population; i.e. a proportion  $h^2\sigma_p^2/\sigma^2$  of the progress previously made.

## V. DISCUSSION

The genetic variability displayed by quantitative characteristics in natural populations of cross-breeding species must in the main be preserved by three types of mechanism. We may expect that there will be some loci affecting the particular metric character concerned which have no influence on reproductive fitness other than that due to the causal relation between fitness and metric deviation. It is almost certain that, in addition, natural selection will favour the heterozygous genotypes at some loci at the expense of the homozygous, resulting in stable intermediate gene frequencies. And, finally, it is possible that some of the genetic variation shown by a given quantitative character is promoted by loci at which deleterious recessive alleles are maintained by recurrent mutation.

As a result of the preceding analysis, we are in a position to compare the effects of loci of the first two categories when artificial selection is imposed on the population. Robertson (1956) has shown that if all the genetic variation shown by an additive character is due to loci showing heterozygote superiority in fitness, a change in the mean of the population of  $x_0$  under artificial selection can be expected to lead to a decline in the relative fitness of the population of  $\bar{S}x_0^2/2h^2\sigma_p^2$ . In this expression,  $\bar{S}$  is a constant called the homeostatic strength of the character concerned, and is related to the mean fitness of homozygotes compared with that of heterozygotes at the individual loci. On relaxation of selection, the return of the mean in one generation is expected to be a proportion  $\bar{S}$  of the progress originally made.

We have shown in the present paper that if all loci affecting an additive genetic character influence fitness solely through their effects on the character

itself, the decline in fitness with artificial selection is expected to be  $1 - \exp[-\frac{1}{2}(x_0^2/\sigma^2)]$ . Provided that artificial selection has not been pushed too far, and that the intensity of natural selection against phenotypic extremes is low, this expression is roughly equal to  $x_0^2/2\sigma^2$ . On relaxation of selection, the change in the mean of the selected population in the first generation is expected to be a proportion  $h^2\sigma_p^2/\sigma^2$  of the progress previously made.

Both models therefore lead to the same pattern of behaviour under artificial selection and relaxation. The decline in the relative fitness of the population (which can be measured satisfactorily in *Drosophila melanogaster* by competition with marked laboratory stocks) is expected to be  $x_0^2/2h^2\sigma_p^2$  times the proportion of the response to selection which is lost after one generation of relaxation, irrespective of the relative importance of the two types of mechanism in maintaining the equilibrium genetic variation. The change in population mean on relaxation of selection is a function both of the homeostatic strength of the character, and of the intensity of natural selection against extreme metric deviation, but the two components cannot be separated.

Waddington (1957) has stressed the importance of distinguishing between these two aspects of the relationship between a quantitative character and natural selection. He has referred to the "true" and "spurious" fitness cross sections of the character in a natural population, corresponding to the model of selection against metric deviation and the homeostatic model respectively. The experimental approach which comes closest to making this distinction possible seems to be a comparison of the effects of relaxation of artificial selection under natural conditions on the one hand, and under controlled environmental conditions on the other. For many characters, one may perhaps attribute the difference in behaviour in the two situations to the effects of natural selection against extreme metric deviation.

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# SELECTION FOR CANALIZATION OF THE SCUTE PHENOTYPE IN *DROSOPHILA MELANOGASTER*

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## Summary

Selection for low variance of scutellar bristle number in scute flies resulted in canalization about a mean of two bristles. Selection for high variance appeared ineffective. The sensitivity of high selection lines to changes in temperature at which the flies were reared was much greater than the sensitivity of the low selection lines and the sensitivity of wild-type cultures.

## I. INTRODUCTION

Waddington (1957) has argued the importance of canalization of developmental pathways. Canalization describes the tendency of a developmental process to hold to its normal course in the face of both genetic and environmental forces tending to deflect it into other channels. The existence of canalization in the development of the scutellar bristles of *Drosophila melanogaster* has been demonstrated by Rendel (1959a, 1959b). The presence of the *sc<sup>sc</sup>* gene segregating in a set of selection lines reduced the number of bristles to about one in scute males and two in scute females. With the reduction in mean phenotype considerable variation appeared between scute flies. This disappeared again when, by selection, the phenotype of the *sc<sup>sc</sup>* genotypes approached four. At the same time variation appeared in *sc<sup>+</sup>* genotypes segregating in the selection lines, when flies with five and six scutellar bristles started to appear as a result of selection for increased bristle number in their *sc<sup>sc</sup>* sibs. It was suggested that the developmental path was canalized at the level of four bristles; if development could be forced out of this path, either up or down, it became more variable. On the other hand, when forced back into a course leading to four as the mean number, variability disappeared again. This variability was at least in part genetic as it responded to selection. So far no account of the production of canalization has been published; it is of interest to show how rapidly canalization can be brought about by appropriate selection.

Falconer and Robertson (1956), by breeding from mice whose weights deviated most from the litter mean and mating heavy to light mice, failed to show that there were any genes controlling sensitivity to the environment. But they did show that when the mean weight started to rise at the eighth generation so did the actual variance. They discount this effect by using the coefficient of variation, which is justified by the correlation of variance with the mean, although, in fact, at low levels of mean this correlation disappears and is reversed. In the line in which they selected for intermediates there are signs of reduction of variance and the possibility that canalization has begun cannot be ruled out. On the assumption that mouse weight is to some extent canalized, one would expect a rise in mean to result in a rise

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in variance, because it takes the phenotype away from the canalized zone; but one would expect any mating system which selected against deviation from the mean to result in a lowered variance, not only because of increased homozygosis but because canalization would be strengthened.

Falconer (1957) also selected for intermediate number of abdominal bristles in *D. melanogaster* without any marked changes occurring in the phenotypic variance or its components. In addition he quoted Harrison (1954, *Drosophila Inf. Serv.* 28: 122-3) as having obtained no change in variance between segments within flies after many generations of selection for both high and low difference between the fourth and fifth abdominal segments. Thoday (1959), who selected for extremes in sternopleural bristle number by disruptive selection, has found changes in variance accompanying changes of mean from the initial value.

In the following description an account is given of a selection experiment in which the character "scutellar bristle number" in *D. melanogaster* was successfully canalized at the level of two bristles. As selection proceeded, the line developed reduced phenotypic variance at 25°C and lowered sensitivity to extreme temperature changes. Attempts to increase variability in a parallel selection line and properties of the scute character in a random-bred Oregon laboratory stock are also described.

## II. EXPERIMENTAL PROCEDURE

The selection lines were derived from a stock homozygous for scute and the blood allele of white. Two lots of 10 single-pair cultures were set up as foundation material. One lot was designated the high line (HL) and in this line the selection procedure was designed to increase the variance of scutellar bristle number. The second lot of 10 cultures was designated the low line (LL) and the procedure for this line was designed to reduce the variance of scutellar bristle number. It was unfortunate that the progeny of the two lines in the first generation had a different variance, but this was in the right direction, so that unconscious selection of the original parents started LL off with a somewhat lower variance than HL.

As the object of the experiment was to see if selection could make a particular phenotype insensitive to both genotypic and environmental influences, the mean bristle number was kept at approximately two bristles. As males have a lower number than females this was not possible by any rigid system of selection. When the number of bristles seemed to be getting too low, males with two bristles were chosen as male parents; when the number got too high, males with one bristle were chosen as male parents. Females with two bristles were used throughout. As we were not primarily selecting for mean expression but for high or low variability of expression about a mean, matings were assessed by calculating the variance of their progeny. The HL parents for the next generation were taken from cultures with the highest variance and the LL parents from cultures with lowest variance. Any females with two bristles and any males with one or two, as the case might be, were selected from the chosen cultures, each of which was always the product of a single mating.

Three pairs of flies were taken from each of the four cultures in HL and LL with the highest and lowest variance respectively. These sets of matings became

lines A, B, C, and D with cultures 1, 2, and 3 in each. Matings in pairs were always made between A♀♀ and D♂♂, B♀♀ and A♂♂, C♀♀ and B♂♂, and D♀♀ and C♂♂. The most extreme culture out of three in each of the sublines A, B, C, and D was chosen on variance each generation. This paper covers the first 28 generations of selection.

From time to time a culture in a subline would fail, leaving only two or even one to be scored. This later became rather commoner in HL than in LL; to avoid losing the opportunity of selecting on variance due to such failures, five cultures

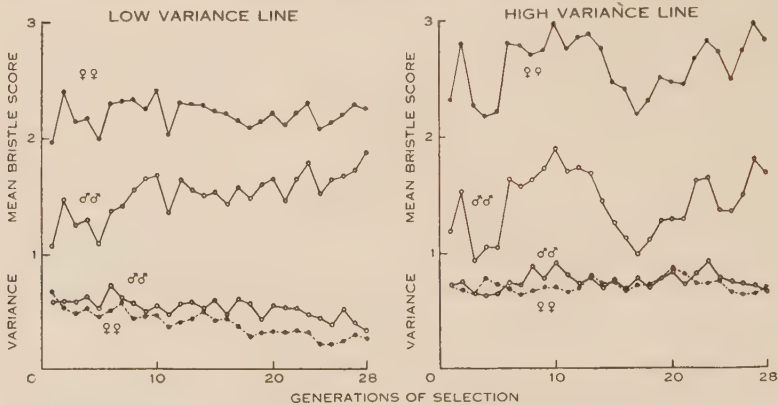


Fig. 1.—Means and variances of the selection lines plotted against generation of selection.

were set up of which the best three were scored. Even so HL sometimes failed to produce three fertile cultures in each subline. There has thus been some selection for fitness over and above selection for variance and this selection has, because of the weakness of HL in this direction, been more severe in HL than in LL. Since parents of intermediate phenotype have always been selected, there should have been a tendency towards homozygosis of additive genotypes making for intermediate bristle number.

After 24 generations HL and LL were compared at different temperatures. Fertile females were allowed to lay eggs for about 12 hr in standard bottles. They were then removed and the bottles put in incubators at 15, 20, and 30°C. As all the selection was carried out at 25°C, the results of the selection lines were taken as typical of this temperature. As flies lay very reluctantly at 15°C the egg laying was all done at 25°C and the temperature treatments lasted from about 12 hr after laying to occlusion. Some wild-type flies were treated in the same way for comparison. They came from an Oregon RC stock which is held in this Laboratory as a wild-type stock.

### III. RESULTS

Figure 1 shows how mean bristle number has changed during the 28 generations of selection. In LL the number has fluctuated about a mean of 2.20 bristles in females; these fluctuations have, if anything, steadied down towards the end of the



experiment. The mean in males has risen steadily from *c.* 1.20 to *c.* 1.80. It is remarkable how much the phenotypic difference between males and females has been reduced during selection. The two are not yet identical as they are in the presence of the *sc<sup>+</sup>* gene but whatever it is that causes the sex difference appears to have far less effect after selection for low variance. The high variance line started with a higher mean; this rose to 1.9 in males and nearly 3 in females at the tenth generation, then fell to 1.0 and 2.2, and rose again to 1.7 and 2.8. There is no sign of any decrease in the sex difference. The within-bottle variances of each generation are plotted in Figure 1 below the means. In LL there is a steady fall in variance to about half the original value in both sexes. Males are more variable

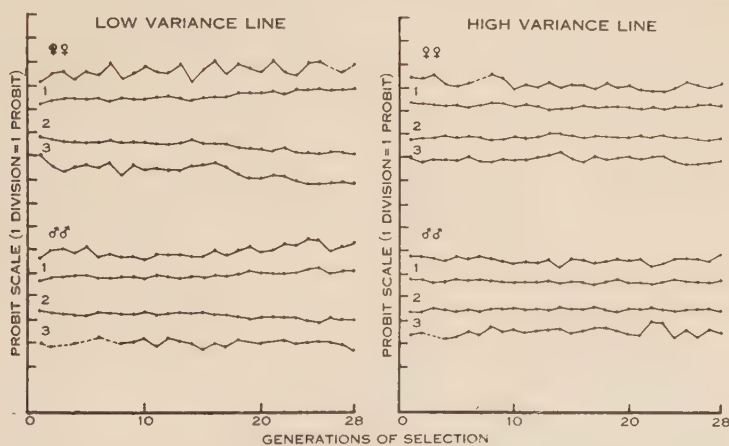


Fig. 2.—Probit distances spanned by the 1-, 2-, and 3-bristle classes in the selection lines plotted against generation of selection.

than females as a rule and there is no indication of variance being proportional to mean. In HL there is no clear indication of any effect of selection. Males tend to be more variable when the mean increases and females when the mean decreases. These tendencies are probably not very significant but are to be expected. As the mean decreases, a high proportion of males come to have 0 bristles and variance tends to drop as there is no class scored below 0; if the mean were reduced till all males had 0 bristles the variance would be 0. As the mean rises a high proportion of females have 4 bristles and, as none have 5, a drop in variance can be expected. The slight difference in mean between LL and HL may in part account for the differences in variance between the lines at the beginning of the experiment.

The effect of selection on the sensitivity of a particular bristle class to influences tending to alter bristle number can best be seen by estimating the distance subtended by the class in standard deviations. The standard deviation is of a distribution, which is described only in part by the bristle scores in most cultures. For if the mean of the distribution falls near the 0 or 4 class large portions of it will lie beyond these thresholds, and there will be no information about such portions since the 0 and 4 class boundaries are the most extreme crossed by either selection line. The method

TABLE 1  
ANALYSIS OF VARIANCE OF SELECTION LINES AT BEGINNING, MIDDLE, AND END OF THE EXPERIMENT

Period	Source of Variation	High Line						Low Line					
		Females			Males			Females			Males		
		D. F.	M. S.	D. F.	M. S.	D. F.	M. S.	D. F.	M. S.	D. F.	M. S.	D. F.	M. S.
Generations 1, 2, 3	Generations	2	33.3	2	37.4	2	14.8	2	14.8	2	13.8	2	13.8
	Cultures within generations	28	3.8	28	3.4	29	2.9	29	2.9	30	2.6	30	2.6
	Within cultures	1386	0.67	1464	0.69	1143	0.50	1143	0.50	1217	0.59	1217	0.59
Generations 13, 14, 15	Generations	2	21.0	2	20.1	2	1.4	2	1.4	2	0.6	2	0.6
	Cultures within generations	30	6.9	30	9.8	31	3.2	31	3.2	30	9.0	30	9.0
	Within cultures	1382	0.78	1353	0.77	1576	0.45	1576	0.45	1594	0.56	1594	0.56
Generations 26, 27, 28	Generations	2	7.4	2	12.0	2	1.2	2	1.2	2	6.5	2	6.5
	Cultures within generations	32	3.8	32	7.4	32	0.6	32	0.6	33	1.8	33	1.8
	Within cultures	1523	0.65	1473	0.72	1708	0.28	1708	0.28	1743	0.43	1743	0.43

and justification of the analysis are dealt with in greater detail by Rendel (1959b). In brief, the distance of the 0/1, 1/2, 2/3, 3/4 cut-off points from the mean of the distribution to which the population measured belongs can be found by calculating the percentage of flies with 0; 0 and 1; 0, 1, and 2; 0, 1, 2, and 3 bristles, and looking for the corresponding probits in Fisher and Yates (1953) tables. By subtraction, the distance occupied by a bristle class in standard deviations can be estimated. Although the distance occupied by the 1-, 2-, and 3-bristle classes can be estimated, that of the 0 and 4 classes cannot as they are incomplete classes, there being no -1 or +5 classes in the selection lines. In the Oregon + stock, however, 3-, 5-, and 6-bristle classes do appear and estimates of the 4 classes can be made.

TABLE 2  
MEAN SCUTELLAR BRISTLE NUMBER IN THE HIGH AND LOW VARIANCE LINES AT  
FOUR TEMPERATURES  
Test at generation 24

Temperature (°C)	High Line		Low Line	
	Females	Males	Females	Males
30	0.75	0.06	1.75	1.12
25	2.71	1.35	2.08	1.52
20	3.16	2.25	2.36	1.89
15	3.74	2.92	2.38	2.12

Figure 2 shows how the distances have changed in the two lines as selection proceeded. The scores of all cultures in a line were pooled each generation before calculating the probit distances subtended by the bristle classes. The difference between culture means, though highly significant, was never very large and it was felt it would not affect the probit scores. The analyses of variance of generations 1, 2, and 3; 13, 14, and 15; and 26, 27, and 28 are shown in Table 1. In generation 26 the probit values were calculated from the mean of the probits of each culture as well as from pooled scores to check the effect of pooling. There was no appreciable difference. It was felt that more might be gained by pooling the data and using large numbers than averaging the probits based on the rather small numbers of each culture and that the time taken in calculating each culture separately was not warranted. In LL the sum of the distances spanned by the 1, 2, and 3 classes has increased steadily; the increase is due almost entirely to the increase in width of the 2 class. In HL there is little regular change. There is a tendency for the total distance spanned to decline in the middle generations. Towards the end of the run the distance increases again. The probit analysis is more informative than the analysis of changes in variance because it is not affected by the extent to which



the distribution tends to approach the 0 or 4 bristle threshold. We expect, therefore, that any effect of selection for high variance will show more clearly on the graph in Figure 2 than in Figure 1. The effect has not been marked and may have been counteracted by selection for fitness. If there is any advantage in a regular development of the scute character, selection for high variance will result in loss of fitness. HL has been far more difficult to keep going than LL. Despite the fact that a subline is represented by five and later six replicates, blanks occur through poor cultures.

TABLE 3  
PROBIT DISTANCES SPANNED BY THE DIFFERENT BRISTLE CLASSES  
IN BOTH SELECTION LINES AT FOUR TEMPERATURES

Selection Line	Bristle Class	Temperature (°C)			
		30	25	20	15
High line Females	1	0·90	0·81	0·75	—*
	2	1·42	1·36	1·38	1·01
	3	—*	1·02	0·88	0·73
Males	1	0·87	0·93	0·81	1·10
	2	—*	1·38	1·08	1·21
	3	—*	1·09	1·04	0·88
Low line Females	1	0·90	1·16	0·66	—*
	2	2·90	2·71	1·99	2·05
	3	0·41	1·32	1·09	1·24
Males	1	0·87	1·29	0·82	—*
	2	—*	2·24	1·59	2·40
	3	—*	0·92	1·10	1·00

\*Indicates incomplete classes.

Table 2 shows the effect of rearing HL and LL at different temperatures. At high temperatures both lines have fewer bristles than they do at lower temperatures but this is far more marked in HL than in LL. In LL there is a difference of about 0.7 bristles in females and 1.0 in males between the mean bristle number at 15 and 30°C, in HL the difference is about 3.0 bristles in both females and males. Obviously selection for invariability has been effective in stabilizing development in the face of a changed environment as well as in a relatively constant environment. An examination of the probit distances occupied by 1-, 2-, and 3-bristle classes at the different temperatures shows that there may be a tendency for canalization to be reduced at lower temperatures. The distances are given in Table 3.

The effect of temperature on Oregon + flies has been investigated and is given in Table 4. Two effects were noticed here. The first is the effect of high temperature

on bristle number which differs considerably from the effect found in scute flies. Extra bristles of the kind found in the earlier selection lines by Rendel (1959a) are large, usually at least half the size of normal ones, and usually near a normal bristle site though sometimes between normal sites. The mean number of such extra bristles is increased slightly rather than decreased at 30°C in the + stock. The second effect is apparently associated with crowding of cultures at 30°C, which results in very small extra bristles always situated between the posterior scutellars.

TABLE 4

FREQUENCY DISTRIBUTIONS FOR SCUTELLAR BRISTLES IN OREGON WILD-TYPE FLIES AT FOUR TEMPERATURES

Number of flies with very small extra bristles situated between the posterior scutellars is given in parenthesis

Temperature (°C)	Females				Males			
	No. of Bristles:				No. of Bristles:			
	3	4	5	6	3	4	5	6
30	—	557	6 (99)	4 (28)	1	612	5 (19)	— (5)
25	1	788	7	—	2	823	—	—
20	1	533	13	1	3	694	2	—
15	1	443	16	2	4	515	2	—

This was an unexpected finding and the details have not yet been worked out. The number of flies of this type are given in parenthesis in Table 4. Table 5 tends to confirm the suggestion that canalization is reduced at low temperatures. Non-scuta flies, in general, are far less susceptible to the effect of temperature than either HL or LL, presumably because they are much more strongly canalized. The 4 zone in the ++ flies of the Oregon stock is about 5 $\sigma$  depending on temperature, whereas in LL the 2 zone is only 2.9 $\sigma$  at best and in HL is about 1.4 $\sigma$ . In other words it takes nearly twice the effort to move across the LL 2-bristle zone that it does to cross the HL 2-bristle zone and four times the effort to cross the Oregon 4-bristle zone.

#### IV. DISCUSSION

Selection has effectively reduced variance in LL and has had little, if any, effect on HL. The change in variance in LL could be due to increased homozygosity; the lack of effect on HL by comparison would be due to selection of heterozygotes from segregating cultures. The experiment of raising the two lines at four different temperatures was undertaken to test this point and it shows that LL is less sensitive

to temperature change than HL. It is difficult to see how homozygosis of LL could account for insensitivity to temperature. If we suppose that the lesser spread of phenotypes in LL at 25°C is due to a higher frequency of homozygous genotypes making for phenotypes with 2 bristles, we must suppose the 0, 1, 2, 3, and 4 phenotypes of the two lines to be genetically similar, though of different frequency. In this case HL should overlap LL at both ends of the scale at all temperatures, as the selection programme has left it with a wider range of phenotypes than LL at 25°C.

TABLE 5  
PROBIT DISTANCES SPANNED BY DIFFERENT BRISTLE CLASSES  
IN THE OREGON + STOCK AT FOUR TEMPERATURES

Temperature (°C)	Females		Males
	4 Class	5 Class	4 Class
30	(5.02)*	0.35	5.34
25	5.38	—	(5.92)*
20	4.87	0.95	5.40
15	4.62	0.87	5.09

\*Indicates incomplete classes.

But this does not happen. At 30°C HL overlaps LL at one end of the scale and at 15°C at the other. Whatever may have been the change in genotype in the two lines since selection started, the genotypes in LL are much less sensitive to temperature change and it seems probable that the bunching of phenotypes due to selection around 2 bristles at 25°C is due to selection for insensitivity of the phenotype to changes in both genotype and environment, which has brought about a situation similar to that found for the 4-bristle phenotype in unselected stocks.

When we attempt to explain the resistance to temperature change in terms of the stability of phenotype at 25°C we find the resistance to temperature change in LL is greater than we expect. The relative variability of the two lines has been assessed by converting the frequencies of the phenotypic classes into a probit scale and comparing the probit distances occupied by the corresponding phenotypic classes. On the assumption that the basic variables responsible for phenotypic differences are distributed similarly in the two lines, the probit distance occupied by a class reflects the extent to which changes in the basic variable will change the phenotype of the class. Thus in LL the 2 class occupies 2.7 probits in females and 2.2 in males whereas in HL it occupies 1.4 and 1.4 respectively. This indicates that to move from 1 to 3 bristles takes one and a half to two times as great a change in the basic variable in LL as it does in HL. So that when an external source of variation, in



this case change in temperature, is imposed on the two lines we expect them both to change the same amount on the probit scale; but that this will result in a much bigger phenotypic change in HL than in LL. In fact, the change on the probit scale is very much greater in HL than in LL; as shown in Figure 3 it is about 2.5 times as great from 30°C to 15°C. In other words our measure of variability indicates that at 25°C, the temperature at which the two lines were selected for different sensitivity, the difference in sensitivity is less than at higher or lower temperatures.

There seem to be two possible lines of explanation for this lack of fit between sensitivity within a restricted temperature range and sensitivity to large changes in temperature. These we shall mention briefly but we have no data adequate to test

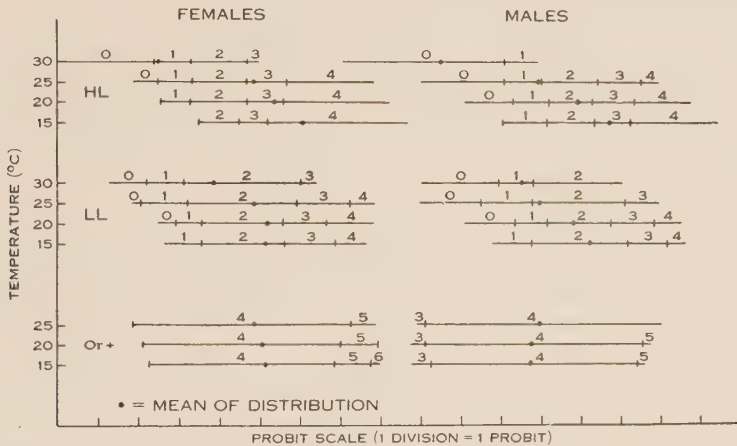


Fig. 3.—Temperature effect on the distribution of the number of scutellar bristles of HL, LL, and Oregon wild-types flies. Distances spanned by the different bristle classes (0, 1, 2, 3, 4, 5, 6) given in probits.

either as yet. The first is that the probit scale of HL cannot be equated to that of LL because the amount of basic variability in the two lines is not the same. It will be remembered that we selected not only for high and low variance but also for intermediate expression and this is expected to lead to some degree of homozygosis (e.g. Robertson 1956). It is possible that in HL the contraction of probit distances in the earlier generations really did reflect an increase in sensitivity but that this has been counteracted by a steady decrease in genetic variance due to selection for intermediates; this selection will have increased in intensity as variability of genetic deviants became phenotypically more obvious. It is possible that in LL selection has also resulted in approach to homozygosis but that selection has decreased in intensity as genetic deviants have come to have a smaller phenotypic effect. The end result might be that the amount of genetic variance in HL has become negligible whereas in LL it is still high. Thus the two probit scales would not be directly comparable as they would measure different things. To account for the results we have found, we have to suppose one scale spans at least twice the variability of the other; to account for this on the ground that one line has lost all its genetic

variance we should have to suppose that at least 60 per cent. of the within-temperature variance was genetic and that HL now has no genetic variance left, but that LL still has much the same as at the start. The second line of explanation would be that the response to temperature was to some extent independent of the factors we have tried to analyse here. It will be noticed that the degree of canalization, measured by the width of the 2-bristle zone, tends to be reduced as temperature falls. It is possible there are other effects which do not correspond to variability of the type taking place within a narrow temperature range.

The effect of temperature differences on a non-scute stock has not added much information which assists the interpretation of the effects on the selection lines. In general, the results can be taken as following the same trends. The ++ line is more strongly canalized and has a smaller temperature effect. It could also be expected to have more genetic variance as it has not been selected in the recent past in a way expected to result in homozygosis. The fact that its response is less than that of LL even on the probit scale could be either associated with the greater strength of canalization or due to the fact that the probit analysis is based on a greater amount of variability.

Whatever the explanation of the discrepancy between the reduction of variation within lines and their sensitivity to temperature, the selection experiment has shown that variation can be reduced by selection and that this reduction of variation is not accounted for by homozygosis. Further, invariability at the temperature at which selection was carried out has been accompanied by a surprisingly large increase in insensitivity to changes in temperature. The invariability produced by selection, therefore, is general and not specific to the variation brought about by a particular set of genes. Finally, invariability at one temperature has resulted in invariability at all temperatures in which the flies were tested.

In a previous paper (Rendel 1959b) genetic dosage was measured on a probit scale and plotted against phenotypic change. The relationship between genotypic and phenotypic change was calculated from the curve and it was suggested that heritability measurements and predictions could be corrected by using such a relationship. It was assumed that environmental effects would follow the same relationship. That is to say, an environmental change would have a phenotypic effect at one level of expression of the scute phenotype, which would be related to phenotypic effects at other levels by the same curve as that describing genetic changes. The low line in that study was reared at about 30°C in order to depress the scute phenotype to a level where at least some *sc<sup>sc</sup>sc<sup>+</sup>* flies would have only three bristles. Despite the differences in temperature and the differences in direction of selection the effect of gene substitutions measured in probits was the same in both lines indicating that this method of measuring gene dosage had some generality. Had there been interaction between gene dosage on the probit scale and temperature this would not have been so. On the phenotypic scale there is very considerable gene-temperature interaction due apparently to the fact that temperature, like genotype, is far more effective on some potential phenotypes than on others. However, in the lines reported in this paper, where selection has been for variability and intermediate expression instead of extreme expression only, the two lines are not

comparable. This may indicate a true gene-environment interaction between the genotypes of the two lines and temperature or that the probit scale has a different value in the two lines owing to reduction in the basic variables of one of them. Despite this inconsistency between lines it can be seen that there is some consistency within lines at different temperatures, for differences between estimates of canalization at different temperatures, though perhaps there, are trivial compared to differences between lines.

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# SELECTION FOR AN INVARIANT CHARACTER, VIBRISSA NUMBER, IN THE HOUSE MOUSE

## II. LIMITS TO VARIABILITY\*

By A. S. FRASER† and B. M. KINDRED†

[*Manuscript received September 11, 1959*]

### *Summary*

A selection experiment for vibrissa number in tabby mice has been extended for 13 generations. Progress of selection for the first seven generations has already been reported. The results of the next six generations of selection are given and the whole experiment is considered with regard to (1) the shapes of the frequency distributions for the three genotypes (+, *Ta*+, and *Ta*·) for succeeding generations; (2) limits to phenotypic variation, i.e. canalization mechanisms; and (3) reproductive fitness.

## I. INTRODUCTION

It is difficult to explain the absence of phenotypic variability on any basis other than genetic fixation, yet various workers have shown that lack of phenotypic variability does not necessarily signify an absence of genetic variability. Goldschmidt (1935), Landauer (1958), and others have found that the frequency and type of phenocopies produced by specific environmental shocks vary between different strains, which are, in the absence of such treatment, indistinguishable. Waddington (1953) extended their observations, by selecting on such exposed variability, showing that selection can, if maintained long enough, produce phenotypic variability in the absence of the variability-inducing treatment. Dun and Fraser (1959) have shown that a mutant gene, tabby, has a similar unmasking effect on genes modifying the numbers of facial vibrissae in the house mouse. This character is not completely invariant in wild-type mice (see Dun 1959) but it exhibits a very small amount of variability and is probably as invariable as most characters subjected to a sufficiently close and exhaustive examination. The tabby gene, a sex-linked partial dominant, causes, in addition to a wide range of other effects, a reduction of the number of vibrissae, and a marked increase of their variability. In the presence of the tabby gene, the number of vibrissae appears to be controlled by a simple polygenic system and has a heritability of the order of 40 per cent. (Dun and Fraser 1958). A selection experiment in which the *Ta* gene is kept segregating has been in progress for 13 generations. In this experiment selection has been practised on the vibrissa number of tabby segregants for both increased and decreased number of vibrissae. The + segregants have been examined to determine whether such selection can cause a breakdown of the invariability of vibrissa number in + mice. Dun and Fraser (1959) have reported on the first seven generations of the experiment. They

\* For Part I of the series see *Aust. J. Biol. Sci.* **12**: 506.

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showed that such a breakdown of the invariability of vibrissa number in normal mice had occurred. Their selection experiment has been maintained on the same basis for a further six generations. Data from these are given below and discussed with reference to (1) the breakdown of the invariance of normal mice; (2) the occurrence of limits to phenotypic variability; and (3) the existence of correlated effects on reproductive fitness.

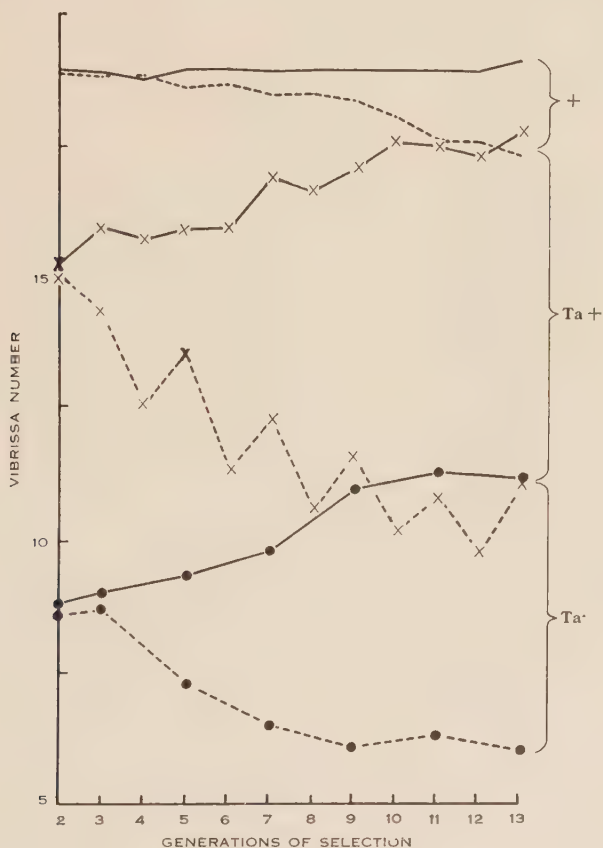


Fig. 1.—Mean vibrissa number at each generation of selection for the three basic genotypes. — High selection line; - - - low selection line.

## II. MATERIAL AND METHODS

These are described in the introductory paper (Dun and Fraser 1959) except for the introduction of a new standard of scoring the postorbital vibrissae in tabby males. These vibrissae are absent in the majority of tabby males; when present they often occur as short, curled, vestigial hairs. Dun considered that such hairs were not true postorbital vibrissae and, therefore, always scored tabby males as lacking the postorbital vibrissae. We now consider that this decision was artificial and have scored all such fibres as true postorbital vibrissae.

An apparent anomaly in the number of generations of selection requires explanation. The experiment has been in progress for 13 generations, yet only the last 11 generations are described and discussed. The reason for this, namely a change in the basis of scoring vibrissa number, which was instituted at the third generation, is discussed in the introductory papers (cf. Dun 1959; Dun and Fraser 1959).

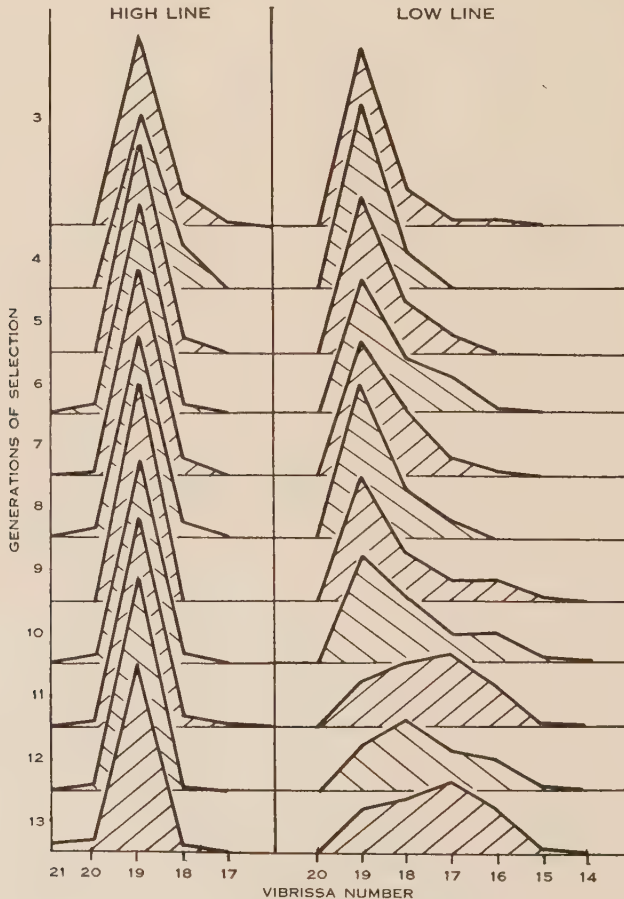


Fig. 2.—Frequency distributions of vibrissa number in + mice of the high and low selection lines.

### III. RESULTS

#### (a) *Effects of Selection on Vibrissa Number*

The segregation of the *Ta* gene produces three main genotypes: + males and females, *Ta*+ (♀), and *Ta*· (♂). The mating scheme is such that *TaTa* females are not produced. Since the tabby gene is a sex-linked partial dominant, this results in three levels of vibrissa number: the + level (c. 19 vibrissae in unselected mice), the *Ta*+ level (c. 15 vibrissae in unselected mice), and the *Ta*· level (c. 8-9 vibrissae in

unselected mice). The mean vibrissa numbers in the high and low selection lines (HST and LST) are shown plotted against generation of selection in Figure 1.

At the  $Ta+$  level of expression, changes of vibrissa number found in the first phase of the experiment (generations 0-7) have continued in the second phase (generations 8-13). There are no indications of a decrease of the response to selection, except in the last two generations of the low selection line, and these are not sufficient to be more than a suggestion. There is a marked alternation of high and low response

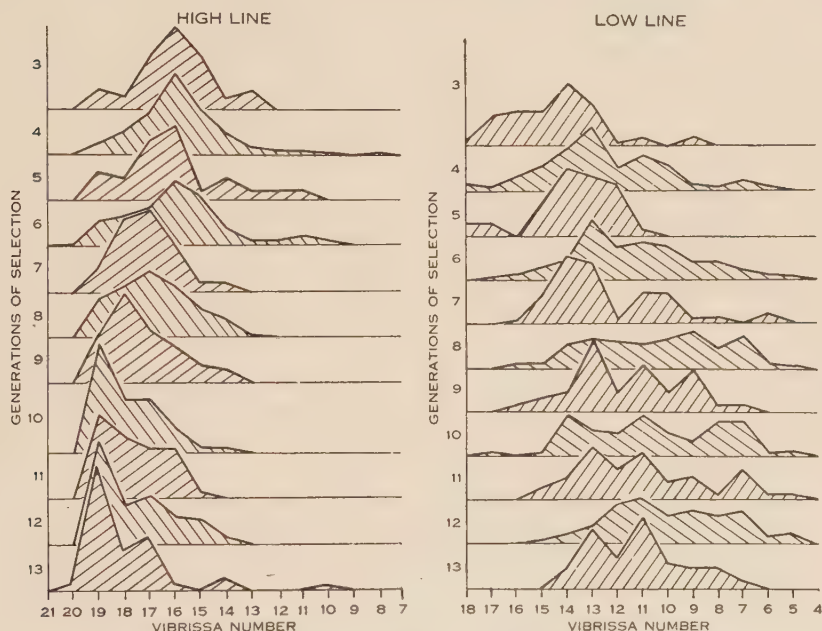


Fig. 3.—Frequency distributions of vibrissa number in  $Ta+$  mice, illustrating the shift of the mode of the distribution towards high vibrissa numbers which occurs in the high selection line as this approaches the “normal” vibrissa number, and the absence of any change in the form of this distribution in the low selection line.

to selection in the low selection line. This is almost certainly caused by the mating system; female parents are  $++$  one generation,  $Ta+$  the next. This indicates that a maternal effect is operating since  $Ta+$  mice produced from  $Ta+$  dams have higher vibrissa numbers than those produced from  $++$  dams.

At the  $Ta\cdot$  level of expression, the response to selection has continued in the high line, but there is a definite decrease in the response to selection in the low line. This may be due to a threshold to vibrissa number at 5, which will be discussed below.

The  $+$  level of expression shows a definite divergence between the high and low lines, which is concluded to be a consequence of the differences of the underlying genotype determining vibrissa number which have been produced by the selection practised at the tabby levels of expression. The low line showed an initially slow response which has markedly increased over the last four generations. Response in the



high line has been slow, but mice have occurred with extra vibrissae at frequencies above those found in unselected normal mice. In the last generation,  $+$  mice with extra vibrissae have been frequent. The frequency distributions of vibrissa number of  $+$  mice in the high and low selection lines are shown in Figure 2.

These distributions show (1) that the distribution of vibrissa number in the LST line has changed markedly from the low variance, high-peaked distribution of the unselected distribution to an almost normal distribution; and (2) that no such change has occurred in the high line. In the high line the only noticeable effect is the formation of a longer "tail" to the higher number.

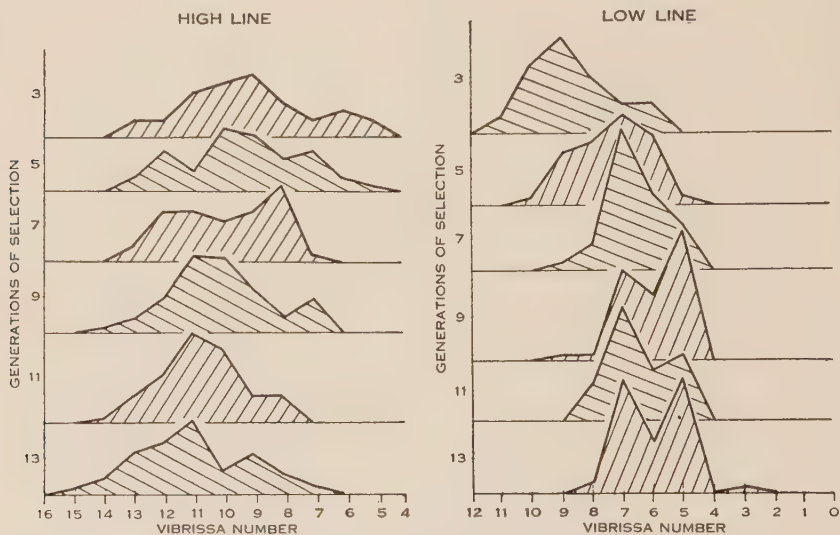


Fig. 4.—Frequency distributions of vibrissa numbers plotted against generation of selection for the  $Ta^+$  level of expression.

The difference between the responses of the two selection lines at the  $+$  level of expression can be due:

- (1) To the different rates of response which have occurred at the  $Ta^+$  level of expression. At this level selection for a decreased number of vibrissae has produced a greater response than selection for an increased number of vibrissae.
- (2) To a stronger canalization of normal vibrissa number at the higher range than the lower. The canalization of normal vibrissa number could be weaker for  $+$  mice with 18–19 vibrissae, than for  $+$  mice with 19–20 vibrissae.
- (3) To the underlying genotype determining vibrissa number being located closer to the lower than the higher edge of the canalization zone. The mean value of the underlying genotype could have a value closer to 18 than to 19 vibrissae. This latter possibility is illustrated in Figure 7.

(b) *Limits to Phenotypic Variability*

The existence of a zone of canalization at 18–19 vibrissae is indicated firstly by the invariance of  $+$  mice, and secondly, by the different rates of response to selection at the  $+$  and  $Ta$  levels of expression. Dun and Fraser (1959) suggested that the tabby gene acts on the canalizing mechanism reducing its limitation of phenotypic variability. On this hypothesis we would not expect any limitation of phenotypic variability to occur in  $Ta+$  mice whose vibrissa number tends towards the normal number. The frequency distributions of vibrissa number of  $Ta+$  mice show a definite shift of the mode of the frequency distribution of vibrissa number towards the high vibrissa numbers in the high selection line as this approaches the “normal” vibrissa number of 18–19. This is illustrated on the left of Figure 3. The analogous data from  $Ta+$  mice of the low selection line (Fig. 3, right) shows that no similar shift of the mode occurs.

TABLE 1  
NUMBERS OF  $Ta\cdot$  AND  $+$  MICE PRODUCED IN THE MATINGS OF  $Ta+$   
AND  $+$  MICE OVER ALTERNATE GENERATIONS OF SELECTION

Generation	Low Selection Line		High Selection Line	
	$Ta\cdot$ ( $\sigma\sigma$ )	$+$ ( $\sigma\sigma$ )	$Ta\cdot$ ( $\sigma\sigma$ )	$+$ ( $\sigma\sigma$ )
3	29	41	34	41
5	34	39	41	48
7	34	53	37	58
9	38	40	58	40
11	47	46	44	56
13	46	48	59	43

Clearly, the original hypothesis, that the tabby gene reduces or removes canalization is not valid, since the above data demonstrate that the zone of canalization at 18–19 vibrissae can be detected in  $Ta+$  mice when their mean vibrissa number is sufficiently high. The correct explanation is that the tabby gene reduces vibrissa number to a point where this is not affected by the zone of canalization, i.e. the explanation suggested by Rendel (1959).

A secondary zone of canalization at a low number of vibrissae is suggested by the decreasing rate of response to selection which occurs in the low selection line at the  $Ta\cdot$  level of expression (see Fig. 1). The presence of such a secondary zone of canalization has been suspected from the absence of  $Ta\cdot$  mice with less than five vibrissae. This deficiency has become increasingly apparent as the frequency distribution of vibrissa numbers in  $Ta\cdot$  mice approaches the lower numbers, since a more and more marked skewing of the distribution occurs. This is shown in Figure 4.

This secondary zone of canalization is restricted to three types of vibrissa: the ulnar-carpals, the inter-ramals, and the supra-orbitals. At each of these positions it is extremely rare for all vibrissae to be absent. Complete loss of all postorals and postorbitals is not rare.

The decreasing rate of response, and the marked skewing of the frequency distribution of vibrissa number in *Ta*· mice of the low line could be due to inviability of *Ta*· mice with less than five vibrissae. That this is not so is shown by the relative frequencies of *Ta*· and + mice in the various generations of selection (see Table 1). We would expect a decreased frequency of *Ta*· males in later generations of selection if there was a decreased viability of such males which had five or less vibrissae. The data show an opposite trend; the frequency of *Ta*· males increases in the later generations of selection.

TABLE 2

MEAN LITTER SIZES OF THE HIGH AND LOW SELECTION LINES AT EACH GENERATION OF SELECTION

Selection Line	Generation of Selection:											
	3	4	5	6	7	8	9	10	11	12	13	
High	6.5	8.0	6.5	7.2	5.4	6.5	6.1	6.2	6.8	6.1	6.8	
Low	5.9	7.2	5.9	6.7	5.3	7.0	5.6	5.5	6.4	5.2	5.7	

*(b) Reproductive Fitness*

A usual concomitant of any long period of selection is decreased reproductive fitness. The mean litter sizes of the high and low selection lines are given in Table 2. There is no indication of a decrease of litter size in the later generations of selection. It is possible that a subdivision of the data on the basis of vibrissa number may show a correlation of reproductive fitness with vibrissa numbers.

TABLE 3

MEAN LITTER SIZES OF THE HIGH AND LOW SELECTION LINES, FOR THE *Ta*+ × +· MATINGS, SEPARATED ON VIBRISSA NUMBER OF THE *Ta*+ PARENT

Selection Line	Vibrissa Number of <i>Ta</i> + Parent:															
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Low	3.5	4.8	5.9	5.6	6.6	5.7	6.8	6.0	6.4	4.6	6.2	6.0				
High											5.2	7.1	7.0	5.8	7.3	8.0

The mean litter sizes of *Ta*+ × +· matings are given in Table 3. Matings have been grouped according to the vibrissa number of the *Ta*+ parent. There is a slight indication of a decrease of litter size from parents with a very low vibrissa number, and of an increase of litter size from parents with a very high vibrissa number. This

indication is very slight and, without more extensive data, does not justify further discussion.

The mean litter sizes of  $++ \times Ta\cdot$  matings are shown in Table 4. Litter size decreases as the vibrissa number of the  $Ta\cdot$  parent increases and decreases away from the mean vibrissa number of nine vibrissae. A number of  $Ta\cdot$  males from the high selection line were crossed to random-bred  $++$  females of another stock. The mean litter sizes of these matings are given in Table 5. There is a far more marked

TABLE 4

MEAN LITTER SIZES FOR THE HIGH AND LOW SELECTION LINES FOR THE  $++ \times Ta\cdot$  MATINGS, SEPARATED FOR THE VIBRISSA NUMBER OF THE  $Ta\cdot$  PARENT

Selection Line	Vibrissa Number of $Ta\cdot$ Parent:									
	5	6	7	8	9	10	11	12	13	14
Low	5.6	6.5	6.4	6.8	6.3					
High					8.0	7.8	7.3	6.8	6.0	6.7

decrease of litter size in matings whose  $Ta\cdot$  parent had a vibrissa number deviating from nine vibrissae. Although the relationship between vibrissa number and litter size requires further clarification, it is clear that there is an association between the vibrissa number of  $Ta\cdot$  males and the size of their litters but no, or only a slight effect, in litters of  $Ta+$  females.

TABLE 5

MEAN LITTER SIZES FOR THE MATINGS OF  $Ta\cdot$  MALES FROM THE HIGH SELECTION LINES TO AN UNSELECTED SAMPLE OF  $++$  FEMALES FROM AN INDEPENDENT RANDOM-BRED STOCK

Vibrissa number of $Ta\cdot$ parent	9	10	11	12	13	14
Mean litter size	6.9	6.2	4.1	4.7	4.3	2.1

#### IV. DISCUSSION

The results show the existence of three systems affecting vibrissa number. These are:

- (1) The genotype on which our selection has been effective. This is termed the "basic" genotype.
- (2) The mutants which modify the effect of the basic genotype. We have considered only the tabby gene. There are two other known mutants which affect vibrissa number. These are the crinkled and ragged mutants. It is



conceivable that these are a part of the basic genotype, and have been separated from it solely by their having marked effects on vibrissa number. They are termed the "mutant" genotype.

- (3) The genetic system which determines the relationship of the basic and mutant genotypes to vibrissa number. This system by its variation of this relationship from simple linearity causes a canalization of vibrissa number, and is therefore termed, the "canalization" genotype.

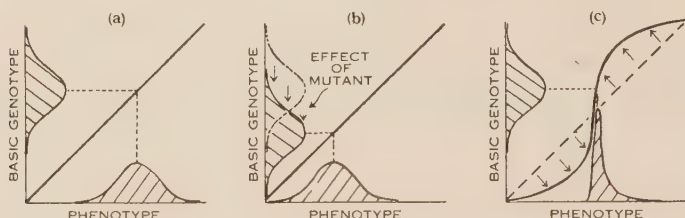


Fig. 5.—Relation of the "basic" genotype to vibrissa number: (a) in the absence of other genetic systems; (b) in the presence of the "mutant" genotype; (c) in the presence of the "canalization" genotype.

The basic genotype is assumed, as a starting point for the construction of our model, to be a simple additive polygenic system. In the absence of other genetic system, the relation of the basic genotype to vibrissa number is assumed to be linear. This is illustrated in Figure 5(a). This can be extended to include the mutant genotype which causes a marked reduction of the number of vibrissae. This effect is assumed to be linear. It is illustrated in Figure 5(b).

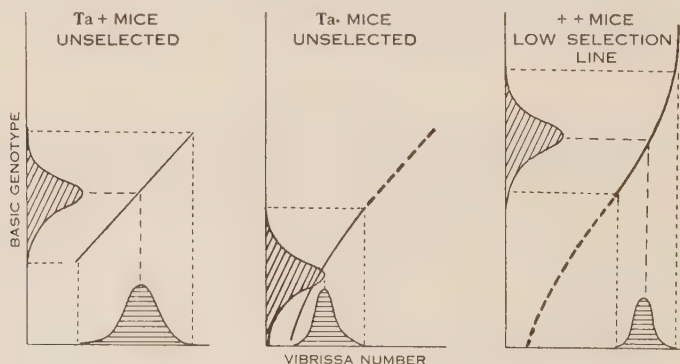


Fig. 6.—Relationship of the "basic" genotype to vibrissa number: (a) in unselected  $Ta+$  mice; (b) in unselected  $Ta-$  mice; (c) in  $++$  mice of the low selection line.

The canalization genotype determines a non-linear relationship of the basic genotype to vibrissa number, such that a large fraction of the possible genotypes produce the same vibrissa number. This is illustrated in Figure 5(c).

If we assume that the basic genotype and the mutant genotype are linear in their relation to vibrissa number, it is possible to estimate the curvilinearity of this

relation introduced by the canalization system. The distributions of vibrissa number in  $Ta+$  mice at the commencement of selection are symmetrical, and extend over a greater range than any other genotype. It is assumed that the basic genotype is, in  $Ta+$  mice, unaffected by the canalization system, i.e. this distribution is assumed to be the result of a linear relation between the basic genotype and vibrissa number. This is shown in Figure 6(a) for model distributions.

This relationship can be extended to include the  $Ta\cdot$  genotype. At the commencement of selection, the distributions of vibrissa number are similar to those of  $Ta+$  mice, being symmetrical and extending over a similar range. This extension is shown in Figure 6(b).

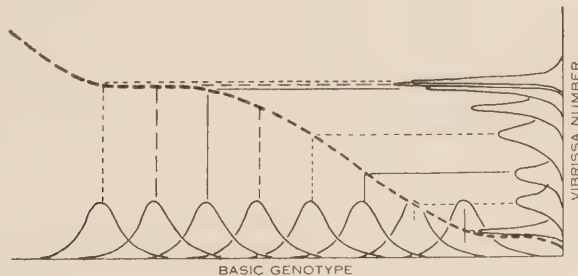


Fig. 7.—Relation of the "basic" genotype to vibrissa number which is concluded from our data, showing how a series of symmetric, equal-spaced distributions of basic genetic variation can result in asymmetric and unequal spacing of distributions of vibrissa number.

A further extension to include the  $++$  genotype introduces the curvilinearity of the primary canalization zone centred on 19 vibrissae. Consider first, the last generation of selection in the low line. The  $++$  mice of this generation have a distribution of vibrissa number which is symmetric, but extends over a much smaller range than of  $Ta+$  and  $Ta\cdot$  mice. This indicates that the relationship of the basic genotype to vibrissa number does not have any marked curvilinearity over this range, but has a steeper slope. This is illustrated in Figure 6(c).

Two other additions can be made. These both show marked canalization. They are of  $++$  unselected mice and of  $Ta\cdot$  mice from the low selection line. In the first, the distribution has a very small range (from 18 to 20) showing that the relation of the basic genotype to vibrissa number is very steep over this range. In the second, the distribution is markedly skewed and has a small range (from 5 to 8), indicating that the relationship is steeply sloped at 5 vibrissae. These aspects are included in the complete model shown in Figure 7.

Two assumptions have been made in the construction of this model which are difficult to demonstrate. These are (1) the independence, genetically, of the three genetic systems, i.e. the basic genotype and the mutant genotype are assumed to have no effect on the canalization of vibrissa number; and (2) that the form of the distribution of the basic genotype is unaffected by selection. This latter assumption is not unreasonable, and examination of the distribution of vibrissa number in  $Ta+$

mice of the low selection line indicates that here, where the range of vibrissa number does not extend into a canalized zone, no marked changes of the form of the distribution have occurred even though a marked change of the mean has been produced by selection.

Our results with vibrissa number and the tabby gene are closely paralleled by Rendel's (1959) results with the number of scutellar bristles and the scute gene in *Drosophila*. One difference is our demonstration of a secondary zone of canalization at a lower vibrissa number. Rendel (1959) found that some degree of secondary canalization occurred at 2 and 6 scutellar bristles, but this was slight and minor compared to the primary canalization at 4 bristles. However, he has shown in a further experiment (Rendel, personal communication) that selection can produce at least a partial canalization around a bristle number of 2.

The existence of a canalized phenotype may be due (i) to a selective advantage of that phenotype; or (ii) to regularities in the basic pattern of development of the tissue concerned. Since our data do not indicate any marked selective advantage of particular vibrissa number, the first alternative does not seem very probable. A model for the second alternative can be found in the widespread effects of the tabby mutant, extending over many aspects of the development of vibrissae and coat hairs. If the basic genotype has similarly widespread effects, then it is feasible to suggest that the canalization of vibrissae is a secondary aspect of the regularity of epithelial development. Further work on this system of canalization therefore needs to be concentrated (a) on clarification of the relation of vibrissa number to selective advantage; and (b) on analyses of the pattern of development affected by the tabby mutant.

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# CYSTINE AND GLUTATHIONE REDUCTASES IN THE CLOTHES MOTH *TINEOLA BISSELLIELLA*

By R. F. POWNING\* and H. IRZYKIEWICZ\*

[Manuscript received October 27, 1959]

## Summary

The larva of the clothes moth *Tineola bisselliella* possesses an enzyme which catalyses the reduction of L-cystine by reduced triphosphopyridine nucleotide (TPNH). Extracts of whole larvae reduce up to 14  $\mu$ moles of cystine per g larvae per hr at pH 7.3. Reduced diphosphopyridine nucleotide (DPNH)-linked cystine reductase and DPNH- and TPNH-linked glutathione reductase of somewhat lower activity are also present in the clothes moth extracts.

## I. INTRODUCTION

The reduction of the disulphide bonds of wool has been shown to increase its susceptibility to digestion by proteolytic enzymes (Geiger *et al.* 1941) and it was suggested by Linderstrøm-Lang and Duspiva (1936) that a reductive break of keratin disulphide bonds may be a necessary prerequisite for digestion of wool by insects. Since thiol compounds are efficient reducing agents of disulphide bonds in wool (Geiger, Kobayashi, and Harris 1942) clothes moth larvae were examined for enzymic activity which would produce thiols by reduction of disulphide compounds.

Enzymic reduction of disulphide bonds in biological materials is now known. Glutathione reductase was described in peas (Mapson and Goddard 1951) and in wheat (Conn and Vennesland 1951), and cystine reductase was found in peas and yeasts (Nickerson and Romano 1952). There is also a protein disulphide reductase known (Nickerson and Falcone 1956).

A preliminary note on a reduced triphosphopyridine nucleotide (TPNH)-linked cystine reductase in clothes moth larvae has already appeared (Powning and Irzykiewicz 1959) and further details of cystine and glutathione reductases in this insect are presented in this paper.

## II. METHODS AND MATERIALS

The insects used in this work were *Tineola bisselliella* (Humm.) larvae about 4 weeks old, bred on a diet of casein containing 3 per cent. yeast. Batches of larvae were homogenized in a "Virtis 45" homogenizer in 4 volumes of water or 0.05M tris (tris(hydroxymethyl)aminomethane) buffer adjusted with HCl to pH 7.3, and centrifuged at 30,000 *g* for 30 min before use. Dialyses were carried out against 25 volumes of the same buffer for 20 hr with one or two changes of buffer. All

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operations were carried out in the cold and the extracts were used as soon as possible after preparation.

The activity of disulphide bond reductases was measured in three ways: (1) the disappearance of reduced diphosphopyridine nucleotide (DPNH) and TPNH was measured at 340  $m\mu$ , in special cuvettes for anaerobic conditions, in a Beckman model DU spectrophotometer. The increase in -SH groups was estimated either (2) by a modification of the Grunert and Phillips (1951) colorimetric nitroprusside method, or (3) by a titrimetric method similar to that of Katchalski, Benjamin, and Gross (1957), using phenyl mercuric nitrate. Reactions were carried out at 25°C under anaerobic conditions.

TABLE 1  
CYSTINE REDUCTASE IN NON-DIALYSED *TINEOLA* EXTRACT

Reaction mixture contained 1 ml *Tineola* extract, 1 ml 0.125M tris-HCl pH 7.3, plus additions as indicated; final volume 2.5 ml. Incubated for 2 hr

Additions ( $\mu$ moles)	$\mu$ Moles Cysteine			
	Colorimetric Method		Titrimetric Method	
	Total	Increment	Total	Increment
Boiled extract				
Nil	0.91		1.40	
L-Cystine (4.2)	1.25	0.34	1.40	0
Fresh extract				
Nil	1.14		1.75	
DPN (2.9)	1.14	0	1.75	0
DPNH (2.2)	1.14	0	1.64	0
L-Cystine (4.2)	3.52	2.38	4.16	2.41
L-Cystine + DPN (2.9)	5.04	3.90	5.90	4.15
L-Cystine + DPNH (2.2)	2.13	0.99	2.63	0.88

The values in Table 1 illustrate slight differences between the results from the two methods of -SH estimation. The titrimetric method measures total -SH groups in the reaction mixture, and therefore in the presence of boiled enzyme there was not any increase of -SH groups on addition of cystine. The colorimetric method measures only -SH soluble in the metaphosphoric acid reagent, and the increase of cysteine in the boiled enzyme mixture is explained by the non-enzymic reduction of a little cystine by protein-bound -SH groups. Although the values for the basic reaction mixture from the titrimetric method are higher than those from the colorimetric method (due to protein-bound -SH groups) the net increase of -SH due to reductase action is about the same in both methods. There is a small amount of sulphide produced in the reaction mixtures, probably from cysteine (Powning 1954), and this is not estimated by the colorimetric method. In most

experiments the colorimetric method was used; however, many confirmatory tests were done with the titrimetric method.

The dehydrogenase substrates glucose 6-phosphate and malate were provided in these experiments only in amounts sufficient for the hydrogen transfer reaction, as higher amounts were found to inhibit the cystine reductase.

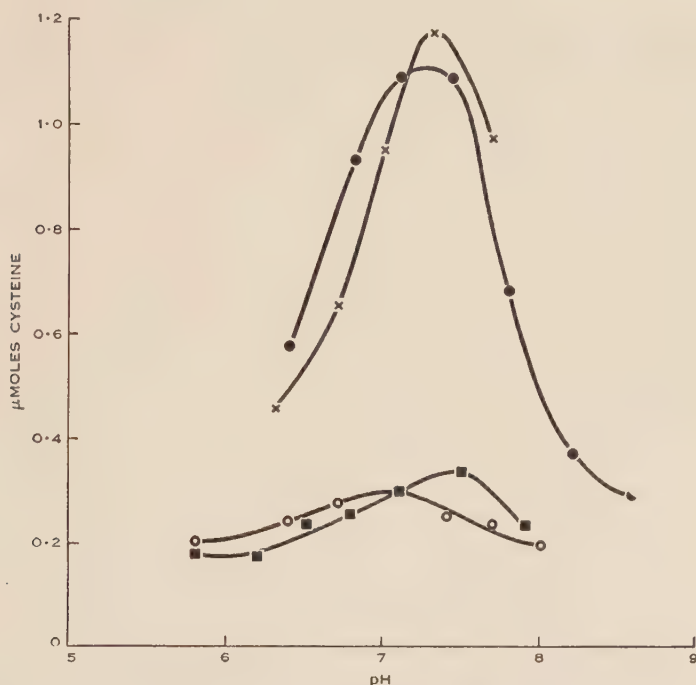


Fig. 1.—Effect of pH on cystine reductase in non-dialysed *Tineola* extract. Reaction mixtures: 1 ml *Tineola* extract, 4.2  $\mu$ moles L-cystine; final volume 2.5 ml. Incubated 2 hr. —SH estimated by the colorimetric method and the results corrected for —SH produced in controls without cystine. Buffers (final concn.):

■ 0.125M Na<sub>2</sub>HPO<sub>4</sub>-HCl; ○ 0.125M Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>-HCl;  
● 0.04M barbitone-HCl; × 0.125M tris-HCl.

Chemical reagents used in this work included diphosphopyridine nucleotide (DPN) (Sigma "90"); triphosphopyridine nucleotide (TPN), 84 per cent. (Sigma); DPNH, 53 per cent. (Sigma); TPNH, 50 per cent. (California Foundation for Biochemical Research); malic acid (B.D.H.); trisodium DL-(+allo)isocitrate (C.F.B.R.); glucose 6-phosphate, disodium salt (Sigma); sodium  $\alpha$ -glycerophosphate (Light); sodium lactate (B.D.H.); L-cystine (B.D.H.); L-homocystine (C.F.B.R.); dithiodiglycollic acid (Light); dithiodibutyric acid (Light). Oxidized glutathione (GSSG) was prepared by aerating reduced glutathione (GSH) (B.D.H.) in an ammonium carbonate solution until the nitroprusside test was negative and the solution was free of buffer.

## III. RESULTS

(a) *Non-dialysed Extracts*

(i) *Effect of pH on Cystine Reduction.*—The enzymic reduction of cystine by non-dialysed extracts of *Tineola* larvae was found to have an optimum pH of about 7.3 (Fig. 1). Phosphate and pyrophosphate caused considerable inhibition of reductase activity and their further use was avoided. Tris buffer at pH 7.3 was used for all experiments.

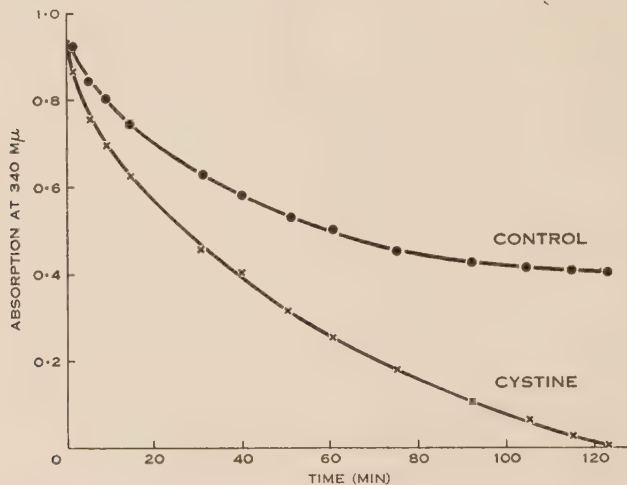


Fig. 2.—Cystine reductase in non-dialysed *Tineola* extract. Reaction mixtures: 1.4 ml *Tineola* extract, 0.54  $\mu$ mole DPNH, 2 ml 0.125M tris-HCl pH 7.3; final volume 3.5 ml. 2.1  $\mu$ moles L-cystine added where indicated. The reaction was carried out in evacuated Beckman cuvettes.

(ii) *Effect of DPNH.*—The cystine reductase of pea seeds and yeasts is DPNH-specific (Nickerson and Romano 1952), and insect tissues were examined for similar activity. A non-dialysed extract from *Tineola* larvae reduced added cystine; however, the addition of DPNH to this reaction mixture resulted in a strong inhibition of reduction and DPN addition caused an activation. The addition of DPN and DPNH to the enzyme without cystine had no significant effect on -SH production. Boiled enzyme was inactive (Table 1).

Spectrophotometric tests of the oxidation of DPNH by *Tineola* extract revealed an activation of this reaction on addition of cystine (Fig. 2). Calculations from the net decrease of absorption at 340 m $\mu$  of the mixture containing cystine show that 0.16  $\mu$ mole DPNH was used per ml of enzyme and this is equivalent to 0.32  $\mu$ mole cystine produced. This is only a fraction of the amount of cystine which is actually formed with the same enzyme preparation under similar conditions (Table 1). Studies with dialysed preparations indicated that endogenous activity of TPNH-linked cystine reductase accounted for this discrepancy.

(b) *Dialysed Extracts*

Attempts to purify the enzyme by acetone and ammonium sulphate precipitation led to greatly decreased activity. However, quite consistent results were obtained with fresh extracts dialysed and used without further treatment or storage.

TABLE 2

DPN- AND TPN-LINKED DEHYDROGENASES IN DIALYSED *TINEOLA* EXTRACT

Reaction mixture contained 0.05 ml dialysed extract, 0.05M (final concn.) tris-HCl pH 7.3 or 9.1, 0.2  $\mu$ mole DPN or TPN, 24  $\mu$ moles substrate; final volume 1.2 ml. 1 unit of activity = change of absorption of 0.001 per min at 340 m $\mu$

Substrate	Activity (units/ml enzyme)			
	pH 7.3		pH 9.1	
	DPN	TPN	DPN	TPN
Lactate	720	44*	360	0
Glutamate	0	0	9*	0
$\alpha$ -Glycerophosphate	35*	0	54*	0
<i>iso</i> Citrate	0	1880		
Glucose 6-phosphate	0	2450		
Malate	1120	1400	3240	900

\* 0.5 ml dialysed extract.

TABLE 3

CYSTINE REDUCTASE IN DIALYSED *TINEOLA* EXTRACT

Reaction mixture contained 1 ml dialysed extract, 1 ml 0.125M tris-HCl pH 7.3, and 0.1  $\mu$ mole DPN or TPN where required plus additions as indicated; final volume 2.5 ml. Incubated for 2 hr. -SH estimated by the colorimetric method

Additions ( $\mu$ moles)	$\mu$ Moles Cysteine		
	Without Coenzyme	DPN	TPN
Nil	0.16		
L-Cystine (4.2)	0.70	0.80	0.82
" + malate (12.5)	5.96	6.30	6.38
" + malate (50)	5.28	5.42	—
" + glucose 6-phosphate (3.1)	5.22	—	6.42
" + <i>isocitrate</i> (20)	3.34	—	3.76
" + $\alpha$ -glycerophosphate (6.3)	0.76	0.96	—
" + $\alpha$ -glycerophosphate (50)	0.64	1.06	—
" + lactate (12.5)	0.70	1.64	—
" + lactate (100)	0.78	2.04	—



(i) *Dehydrogenases*.—Dialysed clothes moth larval preparations were examined for the presence of dehydrogenases which could reduce DPN or TPN. Appreciable malate-TPN, malate-DPN, isocitrate-TPN, and glucose 6-phosphate-TPN activities

TABLE 4  
CYSTINE REDUCTASE IN DIALYSED TINEOLA EXTRACT

Reaction mixture contained 1 ml dialysed extract, 1 ml 0.125M tris-HCl pH 7.3, plus additions as indicated; final volume 2.5 ml. Incubated for 1 hr. -SH estimated by the colorimetric method

Additions ( $\mu$ moles)	$\mu$ Moles Cysteine	
	Total	Increment
Nil	0.06	
L-Cystine (4.2)	0.36	0.30
.. + DPN (0.07)	0.48	0.42
.. + TPN (0.06)	0.48	0.42
.. + DPNH (1.0)	0.75	0.69
.. + TPNH (1.0)	1.73	1.67
.. + glucose 6-phosphate (3.2)	2.76	2.70
.. + glucose 6-phosphate (3.2) + TPN (0.06)	4.09	4.03

TABLE 5  
REDUCTION OF VARIOUS DISULPHIDE COMPOUNDS

Reaction mixture contained 1 ml dialysed extract, 1 ml 0.125M tris-HCl pH 7.3, 4.2  $\mu$ moles disulphide compounds as indicated, 0.06  $\mu$ mole TPN, 3.1  $\mu$ moles glucose 6-phosphate; final volume 2.5 ml. Incubated for 1 hr. -SH estimated by titrimetric method and the results corrected for control without disulphide compound. Different batches of enzyme were used in experiments A and B

Experiment	Disulphide Compound	$\mu$ Moles -SH
A	L-Cystine	5.09
	GSSG	1.63
	L-Homocystine	0.76
	Dithiodiglycollate	0.05
	Dithiodibutyrate	0.17
B	L-Cystine	2.51
	D-Cystine	1.26

were observed at pH 7.3. Reduction of DPN by lactate was moderate at pH 7.3 and 9.1, and very rapid reduction of DPN by malate was observed at pH 9.1 (Table 2).

(ii) *Effect of Dehydrogenase Substrates and Coenzymes on Cystine Reductase.*—In the experiment shown in Table 3 all of the dehydrogenase substrates added increased the activity of the cystine reductase system. Glucose 6-phosphate and isocitrate yield considerable amounts of cysteine, and since these substrates are coupled only to TPN in the insect extracts (Table 2), it appears that TPN is mostly responsible for this reduction. The effect of malate addition supports this, although malate is known to be coupled to both DPN and TPN in clothes moth extracts (Table 2).

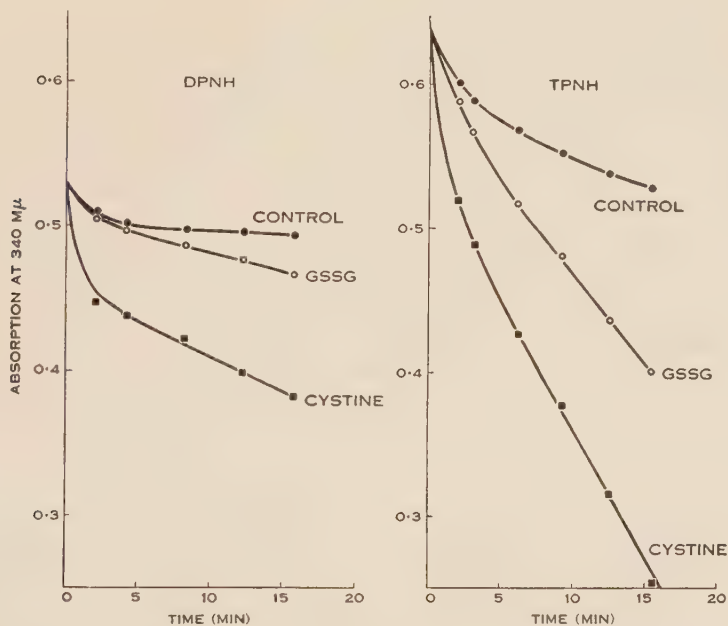


Fig. 3.—DPNH- and TPNH-linked reductases. Reaction mixtures: 1 ml dialysed *Tineola* extract, 0.27  $\mu$ mole DPNH or 0.32  $\mu$ mole TPNH. 2.1  $\mu$ moles GSSG or L-cystine added where indicated, diluted to 3.2 ml with 0.125M tris-HCl, pH 7.3. The reaction was carried out in evacuated Beckman cuvettes.

Further evidence for moderate DPNH-linked and also for considerably higher TPNH-linked reductase activity is presented in Table 4. A slight increase in cysteine formation occurred when cystine or cystine and DPN or TPN were added to the dialysed enzyme; however, DPNH, TPNH, glucose 6-phosphate, and glucose 6-phosphate plus TPN added together with cystine yielded cysteine in increasingly larger amounts. Figure 3 also shows that the cystine-TPNH reductase is much more active than the cystine-DPNH reductase.

(iii) *Course of the Reductase Reaction.*—Figures 4 and 5 show that the TPNH-linked reductase using glucose 6-phosphate as hydrogen donor takes a linear course within certain limits of time and cystine concentration. No evidence was obtained for a reversal of the cystine reductase reaction by attempts to reduce TPN by

cysteine, or to inhibit the oxidation of TPNH by cystine on addition of cysteine. The maximum amount of cysteine observed in our experiments using *Tineola* preparations was about 27  $\mu$ moles per g larvae per hr.

(iv) *Other Disulphide Compounds*.—Table 5 (expt. A) shows that, compared with L-cystine, GSSG and L-homocystine produce considerably less  $-SH$ , and that negligible amounts are obtained from dithiodiglycollate and dithiodibutyrate. The reduction of D-cystine takes place at about half the rate of L-cystine (Table 5,

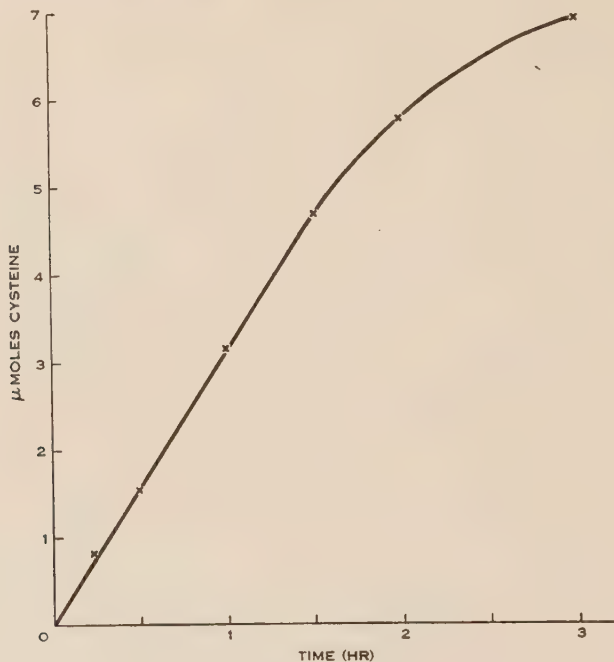


Fig. 4.—Relationship of TPNH-cystine reductase activity of *Tineola* with time. Reaction mixture: 1 ml dialysed extract, 1 ml 0.125M tris-HCl pH 7.3, 4.2  $\mu$ moles L-cystine, 3.1  $\mu$ moles glucose 6-phosphate; final volume 2.5 ml.  $-SH$  estimated by the colorimetric method and the values corrected for controls without cystine.

expt. B). A spectrophotometric test confirms that the reduction of GSSG by TPNH and DPNH is slower than the reduction of cystine. At the same time GSSG is reduced at a higher rate by TPNH than by DPNH (Fig. 3).

#### IV. DISCUSSION

Cystine reductase in pea seeds and yeasts is DPNH-specific (Nickerson and Romano 1952), but it is evident from the present work that clothes moth larvae possess not only DPNH-linked reductase activity but a more active TPNH-linked reductase. The yeast reductase has quite a wide range of optimum pH in phosphate buffer: about pH 6.2–7.3 (Proskuryakov and Buachidze 1956), whereas the clothes moth preparations in tris or barbitone buffers reduce cystine optimally at pH 7.3,

and phosphate and pyrophosphate inhibit the reaction strongly. The equilibrium of the reaction catalysed by the clothes moth cystine reductase appears to strongly favour reduction of cystine, and in this respect it is similar to the reduction of GSSG by animal tissue extracts (Rall and Lehninger 1952).

The insect -S-S- reductases may be coupled *in vivo* to DPN with lactate or malic dehydrogenases, or to TPN with glucose 6-phosphate or *isocitrate* dehydrogenases or with "malic enzyme", all of which have been demonstrated in clothes

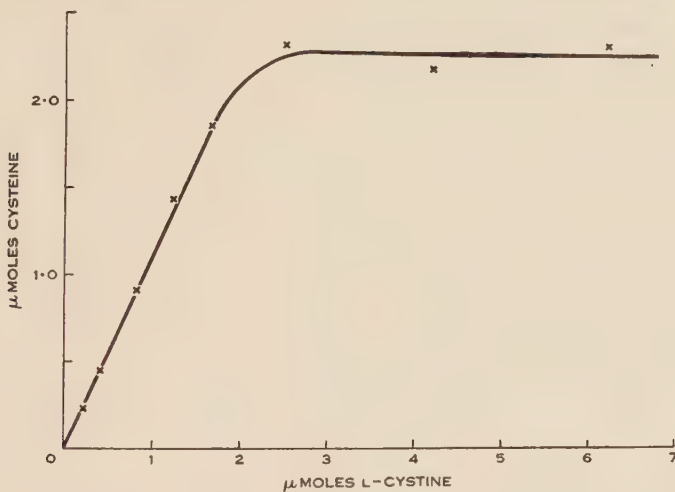


Fig. 5.—Effect of cystine concentration on TPNH-cystine reductase of *Tineola*. Reaction mixture: 1 ml dialysed extract, 1 ml 0.125M tris-HCl pH 7.3, L-cystine as shown, 3.1 μmoles glucose 6-phosphate and 0.06 μmole TPN; final volume 2.5 ml. Incubated for 1 hr. -SH estimated by the colorimetric method and the values corrected for controls without cystine.

moth preparations. It is interesting to note that Proskuryakov and Buachidze (1956) observed a slight stimulation of cystine reductase on addition of malate and DPN to plant extracts, but they did not study the effect of TPN.

The substrate specificity of the dialysed insect preparations is fairly broad; L-homocystine, GSSG, and D-cystine are all reduced at appreciable rates but L-cystine reductase was the most active. Reduction of GSSG by insect preparations is more rapid with TPNH than DPNH (Fig. 3) and this is in accordance with the properties of yeast and liver glutathione reductase (Racker 1955).

Since the extracts used in this work were from whole larvae it is difficult to assign a metabolic role for the cystine reductases. However, any enzyme capable of producing thiol compounds, which could be secreted into the gut of these insects, could play an important part in the process of wool digestion.

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# THE EFFECT OF TEMPERATURE ON THE CHROMATOGRAPHY OF INSULIN ON DEAE-CELLULOSE

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## *Summary*

The effect of ionic strength (range 0.15–0.3), pH (range 7–9), and temperature (range 1–25°C) on the chromatographic behaviour of three samples of insulin on diethylaminoethyl (DEAE)-cellulose columns has been studied. These three factors have a similar effect, a decrease of temperature or pH and an increase in ionic strength lowering the elution volume of the protein. The marked effect of temperature is not due to aggregation–disaggregation of the insulin since bovine plasma albumin which does not aggregate reversibly also showed this effect. The desamido component of insulin could not be detected in commercial insulin under the conditions studied but a minor component varying from 2–6 per cent. of the insulin was separated, as well as various amounts of bound ammonia. Removal of zinc from the insulin did not affect the elution curve.

## I. INTRODUCTION

The effect of aggregation of an acidic protein on its chromatographic behaviour is not known. Since we wished to study the possibility of separating extracted wool proteins by chromatography on diethylaminoethyl (DEAE)-cellulose and it is known that at least one of the components,  $\alpha$ -keratose, is aggregated in solution (O'Donnell and Woods 1956) we have been studying the chromatography of insulin on this basic ion-exchanger. Insulin seemed a suitable model protein since it is an acidic aggregating protein which has been extensively studied by countercurrent distribution (Harfenist and Craig 1952), partition chromatography (Porter 1953), and chromatography on "Amberlite IRC-50" in 8M urea (Cole 1959). This communication describes the pronounced effect of temperature on the chromatography and elution volume of insulin on DEAE-cellulose and also on the non-aggregating system of bovine plasma albumin.

## II. MATERIALS AND METHODS

DEAE-cellulose (Eastern Chemical Corporation) was freed of soluble material absorbing light at 276  $m\mu$  by successive washings on a wrist-action shaker with 1N aqueous potassium chloride solution containing 0.001M "Versene", ethanol (optically pure), 50 per cent. ethanol–water, and 1N potassium chloride until the absorption at 276  $m\mu$  was low and reproducible. For easy filtration on a Buchner it was necessary to remove some of the "fines". Shaking 200 ml of 1N potassium chloride

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with the original DEAE-cellulose (10 g) gave an  $E_{276\text{ m}\mu}^{1\text{ cm}} = 0.55$  but after conditioning as described the  $E_{276\text{ m}\mu}^{1\text{ cm}}$  was only 0.01. The resin was stored in the refrigerator under 1N potassium chloride solution.

The insulins used were the International sample No. 2189 (Anon. 1957) and Boots sample No. 9011G (Boots Pure Drug Co., Nottingham, England) which have been analysed by countercurrent methods (Harfenist and Craig 1952), the latter having been also chromatographed on sulphonated polystyrene resin by Boardman (1955) and the former with column partition chromatography by Porter (1953). Lilly insulin sample No. 535664 (Eli Lilly and Co., Indianapolis, U.S.A.)

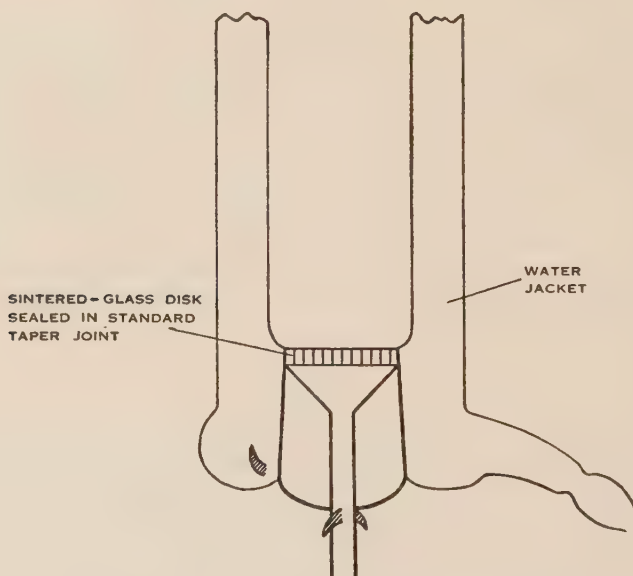


Fig. 1.—Construction of the lower part of the water-jacketed chromatographic columns. A sintered-glass disk was sealed in the top of a standard taper joint with a small built-in funnel to collect the effluent.

and a sample (batch No. A1) from the Commonwealth Serum Laboratories, Parkville, Vic., were also compared. The bovine plasma albumin was obtained from Armour and Co., Chicago, lot No. CA2140.

The jacketed columns (Fig. 1) 0.9 and 3 cm in diameter were maintained at constant temperature,  $\pm 0.1^\circ\text{C}$ , by circulation of water. The area ratio of the cross sections is 11 : 1 and provided this ratio was maintained in scaling up experiments from the smaller to the larger column results from the two columns were comparable. The larger column was used for preparative experiments.

A volumetric fraction dispenser (Simmonds 1958) was used for collecting 3–7-ml fractions but drop counting was found to be the most precise method for collecting fraction sizes of 0.2–0.5 ml when using the smaller column.

### III. EXPERIMENTAL

#### (a) *Preparation of the Columns*

The DEAE-cellulose was washed on a Buchner funnel with the appropriate buffer and after suspending in the buffer the flask was evacuated to remove air bubbles. The slurry was then poured into the column and compacted under 10 lb/sq. in. nitrogen pressure (Sober *et al.* 1956). The columns varied from 12 to 15 cm in length.

#### (b) *Operation and Loading of the Columns*

The columns were eluted with solutions of constant ionic strength and pH, there being no advantage in using gradient elution in this work (Alm, Williams, and Tiselius 1952; Moore and Stein 1956). For the smaller column 0.5 ml of protein solution was added and washed in with  $2 \times 0.25$  ml of buffer. For the larger column these amounts were increased by a factor of 10. During the loading of the column the flow was stopped by putting a finger over the bottom end of the column until the protein or buffer was added. The aliquots were then washed into the cellulose column at the same or a slower rate than was subsequently used for development of the column. This loading flow rate could be controlled by immersing the column in a measuring cylinder of water (Boardman and Partridge 1955), by a rubber tube and clip over the end of the column, or by connecting the buffer reservoir using polythene tubing so that only a small positive head was established before removal of the finger.

Experiments were carried out at either 1, 13, 18, or 25°C. Fractions were collected from the time of application of protein to the column.

The rates of flow of effluent from the columns were varied from 3 to 30 ml/sq.cm/hr with a normal flow rate of 12 ml/sq.cm/hr.

The hold-up volume of a column was determined from the position of the ammonia peak in the insulin samples or by passing arginine through the column since this was not retarded by the resin.

To obtain constant drop size when using the drop counter for collecting fractions it was found that the addition of 0.5 per cent. of a non-ionic detergent solution, "Brij 35" (Moore and Stein 1954), was necessary. Although over the course of an elution the fraction size may have had an overall weight variation of 6 per cent. the difference between any two successive fractions was never more than 1 per cent.

Columns were run without regeneration as long as it was certain all protein had been removed by the eluting buffer. They were equilibrated with new buffer before changing a buffer system. Periodically the DEAE-cellulose was removed, regenerated with 1N potassium chloride, treated with 50 per cent. ethanol-water to eliminate contamination by bacteria or bacterial proteases, and repacked in buffer.

#### (c) *Buffers*

The pH range 7-9 was used and the buffer solutions consisted of 0.001M "Versene", 0.005M tris (tris(hydroxymethyl)aminomethane) plus hydrochloric acid to the required pH, and 0.1-0.3M potassium chloride or other salt. In some



experiments merthiolate (1 in 20,000) was added as a preservative but since this absorbs light strongly at 276 m $\mu$  absorption must be determined by difference from a control tube containing no protein.

#### (d) *Preparation of Protein Solutions*

At the pH values used the insulin would not spontaneously dissolve to give a water-clear solution. It was found preferable to add the insulin to the buffer and then take the pH to 2.3 with 1-6N hydrochloric acid; here the insulin dissolved completely. The pH was then immediately brought back to 7, 8, or 9 with concentrated potassium hydroxide. The various insulin preparations differed in the pH at which they became water-clear on the addition of this alkali. The insulin dissolved between pH 6.8-7.2, the exact value depending on the sample of insulin and this was not altered by the removal of zinc from the insulin (Oncley *et al.* 1952). Furthermore, these solutions were not stable for they often developed cloudiness on standing overnight. For this reason not all samples could be studied at pH 7.

The insulin solution was then either loaded directly onto the column or dialysed against buffer overnight at 2°C on a shaker. 18/32 Visking "Cellophane" tubing was used to dialyse the insulin since Craig, King, and Stracker (1957) have shown that insulin does not pass through this tubing. The concentration of the insulin solution added to the column varied from 5 to 16 mg per ml buffer with a usual working value of 10 mg per ml.

Bovine plasma albumin was dissolved directly in the buffer at a concentration of 4 mg per ml. This caused the pH to fall a little and it was adjusted back to the value of the original buffer with potassium hydroxide solution.

#### (e) *Analysis of Effluent Fractions*

The protein concentration of the effluent from the large column was measured either by absorption of light at 276 m $\mu$  or by taking 0.5-ml aliquots of each fraction and using the ninhydrin technique of Moore and Stein (1948) after adjusting to approximately pH 5 with one or two drops of hydrochloric acid (0.035N). The effluent from the smaller column was analysed by the ninhydrin technique only.

#### (f) *Re-chromatography of Insulin Fractions*

The bulk fractions from the larger column were dialysed on a rocking dialyser at 2°C to free them from salt. The protein solution was concentrated either by freeze-drying or by isoelectric precipitation at pH 5.4. The latter method was preferable in some respects but recovery of the protein was not complete. The recovered protein was then treated in the same manner as the original for chromatography.

#### (g) *Titration Curves of DEAE-cellulose*

Air-dried DEAE-cellulose (0.2 g) was stirred under nitrogen in 20 ml 0.5M potassium chloride (Peterson and Sober 1956), titrated to pH 11 with 1N alkali, and back-titrated in intervals of 0.25 pH units to pH 3 with 1N hydrochloric acid. These titration curves at 10, 20, 30, and 40°C were kindly performed by Dr. H. Lindley on

a pH-stat described by Wood (1959). Equilibrium readings were taken after consumption of acid had ceased for 1 min. No correction was applied for the titration of water.

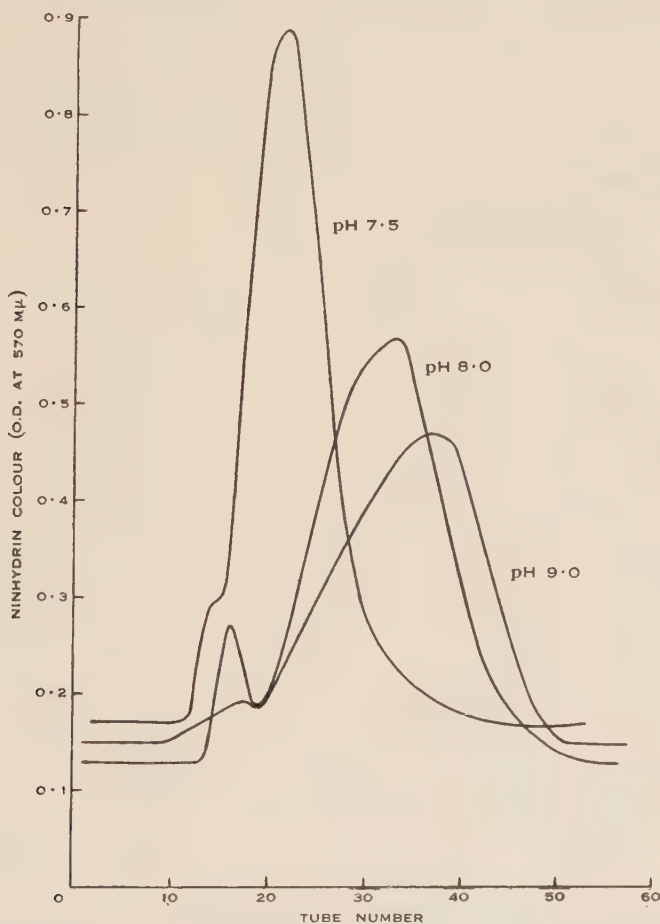


Fig. 2.—Effect of variation of pH at 18°C on the elution curve of International insulin (No. 2189) chromatographed on a  $0.9 \times 13$  cm column of DEAE-cellulose. About 5 mg of insulin was chromatographed in each case, with 0.005M tris buffer containing 0.3M KCl. The effluent was collected in 0.5-ml fractions.

#### IV. RESULTS

##### (a) *Effect of Variation of pH*

The general effect of the variation of pH on the chromatography of insulin on DEAE-cellulose can be predicted from the titration curve of the DEAE-cellulose (Peterson and Sober 1956) and the protein (Cohn and Edsall 1943). Thus at fixed ionic strength a rise in the pH from 7 to 9 increases the charge on the protein causing stronger binding between the protein and cellulose; this is manifest in an increasing elution volume with increase in pH as shown in Figure 2.

(b) *Effect of Variation of Ionic Strength and Nature of Ions*

As shown in Figure 3 the effect of increasing the ionic strength at a fixed pH value is to lower the elution volume. With 0.1M potassium chloride the insulin was retained by the column.

In the chromatography of proteins on negatively charged resins such as "Amberlite IRC-50" multivalent buffer anions such as phosphate have been particularly useful (Hirs, Moore, and Stein 1953; Boardman and Partridge 1955; Moore and Stein 1956) because of combination with the protein and concomitant lowering of

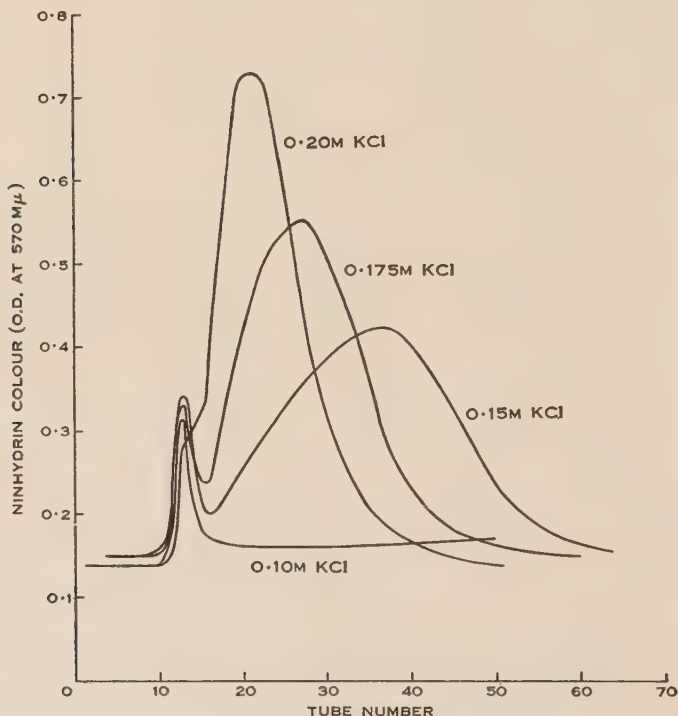


Fig. 3.—Effect of ionic strength on the elution curve of International insulin (No. 2189) chromatographed on a  $0.9 \times 13$  cm column of DEAE-cellulose. About 5 mg of insulin was chromatographed in each case, with 0.005M tris buffer containing varying concentrations of KCl at pH 7.5 and 1°C. The effluent was collected in 0.5-ml fractions.

the isoelectric point. With insulin at pH 8 and 18°C in buffer containing 0.1M dipotassium hydrogen phosphate (ionic strength approx. 0.3) insulin was not eluted from the DEAE-cellulose. When sulphate or acetate (of equal ionic strength) were substituted for 0.3M chloride ion at pH 8 and 18°C the elution curve of the insulin was very spread. For our purposes the combination of chloride ions and tris buffer cations, which have advantages for chromatography on anion exchange resins (Boman and Westlund 1956) proved the most satisfactory.

*(c) Effect of Temperature*

It was found (Fig. 4(a)) that lowering the temperature at which chromatography is carried out has the same effect on the chromatographic behaviour of insulin as increasing the ionic strength or lowering the pH, i.e. the elution volume is lowered. Furthermore, as can be seen from the figure the skewness was changed. The same behaviour was found with bovine plasma albumin (Fig. 4(b)).

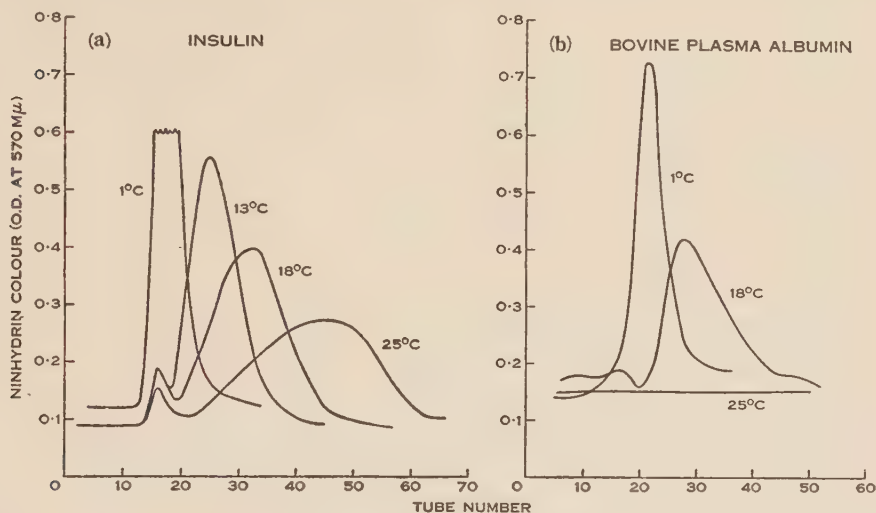


Fig. 4.—(a) Effect of temperature on the elution curve of International insulin (No. 2189) chromatographed on a  $0.9 \times 13$  cm column of DEAE-cellulose. About 5 mg of insulin was chromatographed in each case, with  $0.005M$  tris buffer containing  $0.3M$  KCl at pH 8 as eluent. (b) Elution curves of bovine plasma albumin chromatographed on a  $0.9 \times 14$  cm column of DEAE-cellulose at 1, 18, and  $25^\circ C$ . About 2 mg of albumin was chromatographed in each experiment, with  $0.005M$  tris buffer containing  $0.11M$  KCl at pH 8. The effluents were collected in 0.5-ml fractions.

*(d) Effect of Flow Rate*

The elution curves of insulin developed at the normal flow rate of 12 ml/sq. cm/hr with buffer at pH 8 containing  $0.3M$  potassium chloride at  $18^\circ C$  had a trailing edge which was steeper than the leading edge so that the curves were slightly asymmetric (Fig. 5(b)). This effect was not due to lack of equilibration with the resin since reducing the flow rate to 3 ml/sq. cm/hr increased the skewness (Fig. 5(a)) while increasing the flow rate to 30 ml/sq. cm/hr increased the symmetry with no change in the elution volume.

*(e) Comparison of Insulin Preparations*

Chromatography over the pH range 6.5–9 of the insulin samples studied revealed the presence of three components. The first of these, which was not absorbed on the column, was ammonia. This varied in amount among various insulin preparations. The presence of the ammonia peak on the elution curve could be avoided by (1) measurement of the ultraviolet absorption curve at  $276 m\mu$  on the 5-ml



fractions from the larger column: ninhydrin determinations confirmed the presence of ammonia; (2) dialysis overnight of the insulin sample against the eluting buffer

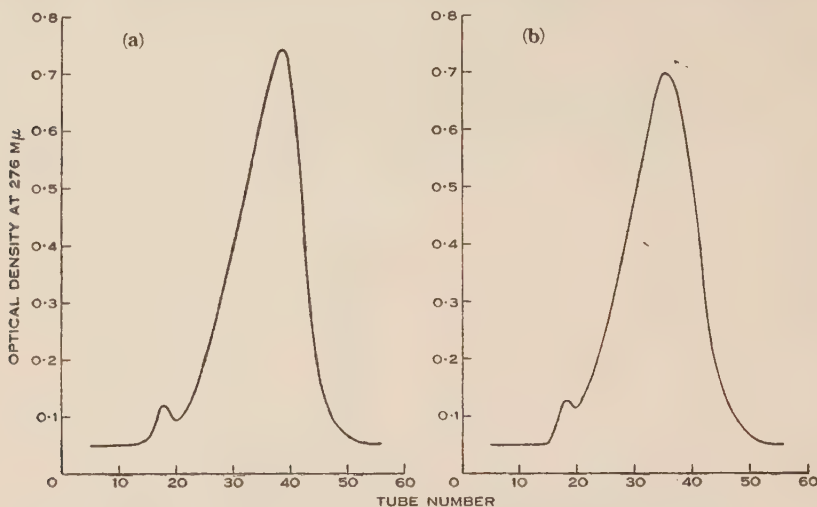


Fig. 5.—Effect of flow rate on the symmetry of the effluent curves of Commonwealth Serum Laboratories insulin chromatographed on a  $3 \times 15$  cm column of DEAE-cellulose. About 60 mg of insulin was chromatographed in each case at  $18^{\circ}\text{C}$ , with  $0.005\text{M}$  tris buffer containing  $0.3\text{M}$  KCl at pH 8 as eluent. The effluent was collected in 5.5-ml fractions. The flow rates were (a) 3 ml/sq. cm/hr; (b) 12 ml/sq. cm/hr.

followed by ninhydrin assay of the tubes (the column was first equilibrated, and subsequently developed with, the dialysate); (3) isoelectric precipitation of the

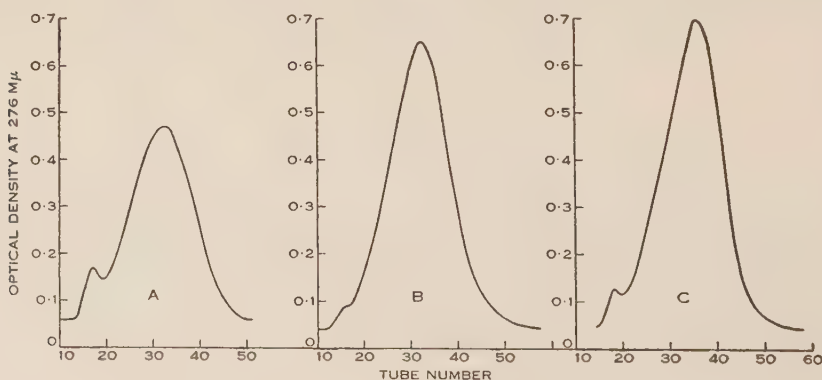


Fig. 6.—Comparison of insulins by chromatography on a  $3 \times 15$  cm column of DEAE-cellulose at pH 8 and  $18^{\circ}\text{C}$  in  $0.005\text{M}$  tris buffer containing  $0.3\text{M}$  KCl. A, 50 mg Lilly insulin; B, 63 mg International insulin (No. 2189); C, 62 mg Commonwealth Serum Laboratories insulin. Effluents collected in 5.5-ml fractions.

insulin solution followed by centrifugation and re-dissolution of the precipitate in buffer, the elution being followed by ninhydrin assay; (4) alkaline hydrolysis of

the fractions before ninhydrin assay (Hirs, Moore, and Stein 1956). The second chromatographic component which overlapped the ammonia peak was a minor one and varied from 2 per cent. in the International sample to 6–7 per cent. in the Lilly sample (Fig. 6). The third and major component is insulin plus any desamido component present. Below pH 7.2–7.3 the tendency of the insulins to come out of solution was reflected in a trailing tail which was more apparent at the lower pH values.

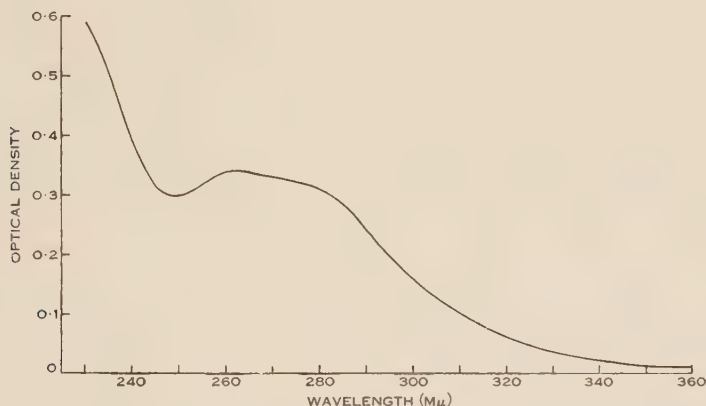


Fig. 7.—Ultraviolet absorption curve of an optically pure ethanol extract of Visking "Cellophane" measured with a Beckman DK-2 recording spectrophotometer.

Removal of the zinc from the Lilly and International samples did not affect their chromatographic behaviour.

The recovery of the ninhydrin-positive material was between 90 and 100 per cent. depending on the choice of base line. The fact that the curve returned to its original base line suggested that all material was removed from the column.

#### (f) *Re-chromatography of Insulin Fractions*

Cuts were made to separate the second and third components of the insulin chromatograms. These fractions were dialysed free of salt and freeze-dried before being dissolved and re-applied to the columns. Unfortunately this process of concentration produced a fast running artifact which was due to non-dialysable material dissolving from the "Cellophane" tubing. For example, 50 cm of "Cellophane" tubing when soaked in 50 ml optically pure ethanol gave the ethanol an optical density of 0.4 at 276 mμ (cf. Fig. 7). Preliminary washing of the "Cellophane" with optically pure ethanol followed by 50 per cent. ethanol-water and water at 50°C before using for dialysis did not completely eliminate the artifact. This artifact, in the case of the third or major component, could be avoided by isoelectric precipitation of the eluate fractions after dialysis followed by centrifugation and re-dissolution of the protein in buffer. It then emerged as a single peak. Such a treatment did not remove the second component from the original insulin.

The second component always re-chromatographed in the same position and was contaminated by this artifact but it did not contain any of the major third component thus eliminating the possibility that it was due to an aggregation equilibrium of the insulin. This second component could not be precipitated at pH 5.4.

## V. DISCUSSION

Previous studies have shown that purified insulin may contain several components. The presence of varying amounts of bound ammonia was shown by Chibnall, Mangan, and Rees (1958) who also found that the only insulin examined by them which gave the theoretical six residues of amide nitrogen (Sanger, Thompson, and Kitai 1955) was a preparation isolated by Harfenist and Craig (1952) following countercurrent distribution. A de-amidated component of insulin was separated by Harfenist and Craig and analysed by Harfenist (1953). More of this component was found in a sample of Lilly insulin than in the Boots sample of insulin (No. 9011G) or International Batch No. 2189.

We have no evidence that we have succeeded in separating the desamido component of insulin on DEAE-cellulose since countercurrent studies by Human and Leach (unpublished data) of the Lilly insulin used revealed approximately 18 per cent. of the desamido component whereas we only found a small component of 6 per cent. separated from the main peak. Moreover, a sample of the desamido insulin isolated by Human and Leach according to the method of Harfenist and Craig (1952) when chromatographed on DEAE-cellulose at pH 8 and 18°C with buffer containing 0.3M potassium chloride gave a major peak in the same position as the original insulin and also a minor peak (12 per cent.) in the same position as the minor peak in the original insulin.

Another component which has been detected in insulin is glucagon (Porter 1953) which is present to the extent of no more than 1 per cent. Because the minor component obtained by us is much more than 1 per cent. of the total insulin it is unlikely to be glucagon. Harfenist and Craig (1952) showed the presence of a third component in insulins and it is possible that this is the component that we have isolated on DEAE-cellulose. We have made no measurements of activities of insulin or its fractions.

Cole (1959) has recently reported the separation of commercial insulins into three components by chromatography in 8M urea at pH 6 on "Amberlite IRC-50" columns. Because of the insolubility of insulin in the pH range 4-7 we have not been able to work near the isoelectric point of insulin (in the absence of urea or dioxan) where minor differences in charge between various proteins might be expected to have their greatest effect in chromatographic separation. From the data available in the literature (Moore and Stein 1956; Hill, Kimmel, and Smith 1959) it appears that most successful chromatographic separations of proteins with similar isoelectric points have been achieved near the isoelectric point of the proteins.

The three variables—temperature, ionic strength, and pH—could be varied independently to produce similar effects on the chromatographic behaviour of insulin on DEAE-cellulose; for example, a variation of any one could be compensated by

adjustment of the other two. The effects of variation of pH and ionic strength are similar to those obtained during chromatography of proteins on this and other resins (Moore and Stein 1956; Sober *et al.* 1956) but to our knowledge the effect of temperature on chromatography on DEAE-cellulose has not previously been emphasized. Most chromatographic studies are conducted at constant temperature, e.g. at 4°C, to minimize denaturation effects. In our preliminary experiments conducted at room temperature a variation of 5°C during the course of the experiment led to breaks and spreading in the effluent curve.

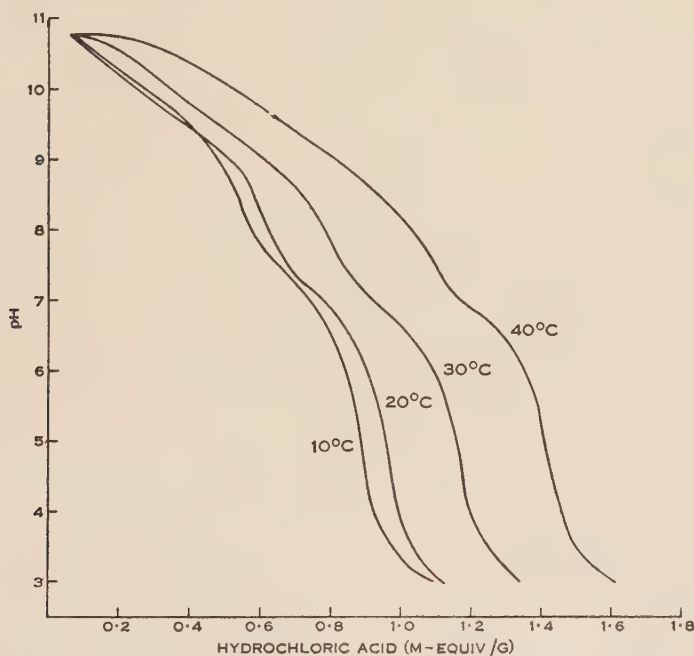


Fig. 8.—Titration curves of DEAE-cellulose in 0.5M KCl at temperatures of 10, 20, 30, and 40°C.

During the chromatography of histones on "Amberlite IRC-50", Crampton, Moore, and Stein (1955) found that a variation in temperature from 4 to 25°C did not affect the effluent pattern. Similarly Boardman and Partridge (1955) found their major peaks were in the same position at 2 and 25°C when chromatographing carbon monoxide haemoglobin on "Amberlite IRC-50" although at the higher temperature more denaturation occurred. The temperature effect observed with insulin on DEAE-cellulose is not peculiar to the aggregating insulin system since it also occurs with the non-aggregating bovine plasma albumin. It is known that the titration curves of insulin do not vary considerably between 4 and 25°C (Cohn and Edsall 1943) and we must consider the possibility of temperature affecting the  $pK$ 's of the amino groups on the DEAE-cellulose.

From the titration curves of DEAE-cellulose (Fig. 8) it is seen that there is a marked effect of temperature on the ionization of the basic groups of this insoluble



polyelectrolyte; as the temperature is increased the number of basic groups on the cellulose at any pH (range 3–11) usually increases. The increase is greater at higher temperatures and explains the marked increase with temperature in the elution volumes of proteins chromatographed on DEAE-cellulose. Moreover, the effect of temperature on the ionization of insulin (Cohn and Edsall 1943), though small, supplements this effect.

#### VI. ACKNOWLEDGMENTS

The authors wish to thank Dr. J. P. E. Human for the construction of a fraction-dispensing apparatus and Dr. H. Lindley for carrying out the titration curves on DEAE-cellulose. We are also indebted to Dr. Human and Dr. S. J. Leach for a sample of desamido insulin.

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# THE ISOLATION AND PROPERTIES OF SOME SOLUBLE PROTEINS FROM WOOL

## I. THE ISOLATION OF A LOW-SULPHUR PROTEIN\*

By J. M. GILLESPIE†

[Manuscript received July 29, 1959]

### Summary

Wool was extracted with 0.1M potassium thioglycollate at pH 11.0 for 2 hr at 50°C and the soluble proteins were precipitated by acidifying to pH 5, redissolved in potassium thioglycollate at pH 10, and reacted with sodium iodoacetate at pH 9 to give a mixture of at least five electrophoretically distinct *S*-carboxymethyl kerateines.

Acidification to pH 4.1 precipitated about two-thirds of the protein, including the major protein, and gave two well-defined fractions. Solution of the precipitate fraction in buffer at pH 11.0 showed, on electrophoresis, one main peak and two smaller peaks. Fractional precipitation of this fraction with acetone gave a low-sulphur protein which gave a single boundary on electrophoresis. The composition and some of the properties of this protein are discussed and compared with those of previously isolated wool proteins.

Previous work in this field has been summarized and a suggested nomenclature for wool proteins isolated by reduction and alkylation is given.

## I. INTRODUCTION

Interest in the proteins from wool is increasing following improvements in techniques for the preparation and characterization of these materials and with the recent increase in knowledge concerning their location in the fibre (Rogers 1959*a*) and their sites of synthesis in the follicle (Ryder 1956, 1958). It is now generally accepted that the wool fibre can be separated into sulphur-rich and sulphur-deficient protein fractions as summarized in Table 1. It is probable that the low-sulphur proteins, as typified by *S*-carboxymethyl kerateine A2 (SCMKA2)‡ and  $\alpha$ -keratose, originate in the microfibrils or  $\alpha$ -filaments which are the fibrous units of structure of wool (Birbeck and Mercer 1957) and that these are embedded in a non-fibrous sulphur-rich matrix from which the high-sulphur proteins can be extracted (Rogers 1959*b*). Recognition that these extracted proteins originate in different histological constituents of the fibre heightens interest in their purification and characterization. Reduction followed by carboxymethylation offers one procedure for the separation of these proteins but much still remains to be done in this field, particularly in the study of the purity of the isolated proteins and of the changes undergone by them during the reduction and alkylation reactions.

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‡ Appendix I presents a summary of the separation of reduced proteins from wool performed in this Laboratory, together with a suggested nomenclature.

Gillespie and Lennox (1953, 1955) isolated a protein from wool by successively extracting Merino 64's top five times with potassium thioglycollate at pH 10·5 and once at pH 11·4. The last extract contained kerateine 2, a low-sulphur protein which was converted to the *S*-carboxymethyl derivative (Gillespie 1956) and purified by fractional precipitation with zinc acetate or ammonium sulphate (Gillespie 1957), giving the protein now termed SCMKA2 (see Appendix I).

TABLE 1  
SULPHUR-CONTAINING FRACTIONS OF THE WOOL FIBRE

Method of Extraction	Fractions Obtained		References
	Low-sulphur Fraction (% S)	High-sulphur Fraction (% S)	
Alkaline thioglycollate	3·3	4·5	Goddard and Michaelis (1935)
Cetyl sulphonic acid	4·2	7·5	Lindley (1947)
Peracetic-ammonia	2·4	6·1	Alexander and Earland (1950)
Chlorine dioxide	1·3	5·3	Speakman and Das (1950)
Alkaline thioglycollate	1·5*	6·5	Gillespie and Lennox (1953); Gillespie (1958)
Peracetic-ammonia	1·9	5·8	Corfield, Robson, and Skinner (1958)
Urea extract of "wool roots"	1·7	4·1	Rogers (1959a)
Performic acid with pH 8 extraction	1·96†	4·72†	O'Donnell and Thompson (1959) and unpublished data

\* As *S*-carboxymethyl cysteine.

† As cysteine acid.

The pH 10·5 extracts were shown to be electrophoretically heterogeneous, and to contain at least five peaks, one of which had a similar mobility to kerateine 2. In a preliminary account, Gillespie (1958) reported the isolation of this material as its *S*-carboxymethyl derivative in a form moving with a single boundary on electrophoresis. In this paper the isolation and properties of this easily extractable low-sulphur protein (SCMKA1) will be described in detail. Together with SCMKA2, the low-sulphur proteins in the pH 10·5 extracts account for about 40 per cent. of the weight of wool. SCMKA1 and SCMKA2 may be different forms of the same protein, but until such time as their true identity has been established it is proposed to keep separate names for them.

## II. MATERIAL AND METHODS

### (a) Preparation of Soluble Wool Proteins

In the original procedure used for isolating kerateine 2 the pH 10·5 extractable material was spread over five fractions, all of which were in dilute solution (Gillespie and Lennox 1953). In the present study, where the isolation of these proteins was

desired and not their complete removal from the wool prior to the extraction of keratine 2, a somewhat different procedure was used. 100 g (air dry weight) of solvent-scoured Merino 64's wool top was soaked for 1 hr at 20°C in 3 l. of 0.1M sodium carbonate of initial pH 11.0. The wool was then roughly dried in a cotton towel and transferred to 3 l. of 0.1M potassium thioglycollate, pH 11.0, at 50°C and kept at this temperature for 2 hr. The final pH was 10.4–10.5 and about 40 per cent. of the wool was dissolved. The solution was filtered, rapidly cooled to 20°C, the pH adjusted to 5, and the precipitated protein was separated by filtration. The precipitate was dissolved in about 1 l. of 0.1M potassium thioglycollate at pH 10 using a Waring Blendor\* modified to prevent foaming. The pH was then adjusted to 9, 40 g of iodoacetic acid (pH 6) was added, and the pH continuously adjusted to between 8 and 8.5 until the reaction was complete, as evidenced by the disappearance of both the ferrous thioglycollate colour in the protein solution and the nitroprusside colour reaction. The pH was then adjusted to 7.0 and the mixture of *S*-carboxymethyl keratines thoroughly dialysed for 24 hr in running tap water. This preparation was used in the studies which follow and will be referred to as SCMK.

#### (b) Spectrophotometry

Extinction curves were determined and measurements were made of the optical density of protein solutions in a Beckman model DU spectrophotometer. Throughout this paper optical density measurements at 278 m $\mu$  will be used to indicate protein concentration even though it is realized that wool proteins have extinction coefficients ( $E_{1\text{cm}}^{1\%}$ ) varying at least between 4 and 9, but in fractionation experiments, where the analytical data on the proteins were not known, this procedure provided a useful approximation allowing rapid measurements to be made on dilute solutions.

Partially precipitated SCMK solutions were often quite turbid, rendering optical density measurements unreliable. This turbidity could be eliminated by making an initial dilution with an equal volume of glacial acetic acid. At 278 m $\mu$  the absorption by acetic acid is small enough to be neglected.

#### (c) Solubility Curves

The effect of protein precipitants on the solubility of these proteins was determined by mixing appropriate amounts of protein solution with precipitant in 40-ml glass-stoppered tubes to give final volumes of 20 ml, after which the tubes were gently rocked at 2°C for 24 hr. In experiments with ethanol–water and acetone–water as precipitants, a temperature of –5°C was used and the concentrations were recorded as per cent. v/v of anhydrous solvent. The insoluble protein was centrifuged off at the equilibration temperature and the concentration of protein in the supernatant was measured spectrophotometrically at 278 m $\mu$ . When acetone was used as the precipitant, this was not possible and the biuret procedure of Mehl (1945) was used instead.

\* The modified Waring Blendor used had an aluminium vessel with screwed lid (Arthur H. Thomas Catalogue No. 4282-D) with a threaded hole in the top of the lid near one edge arranged so that all air could be replaced by fluid and the vessel then completely sealed.



In experiments designed to study the effect of pH on the solubility of SCMK proteins, a somewhat different approach was used, for when acid or buffer was poured into solutions of these proteins a gelatinous lump was formed which came to equilibrium with the system only very slowly. Addition of these reagents by dialysis was less troublesome in this respect. 5-ml aliquots of 0.5 per cent. protein were dialysed in "Cellophane" tubes against 30-ml quantities of buffers of constant ionic strength but varying pH over the range 1–5. These consisted of 0.05M phosphoric acid, 0.1M acetic acid with sufficient sodium hydroxide to give the required pH, and sufficient sodium chloride to give an ionic strength of 0.2. After rocking at 2°C for 24 hr the contents of the dialysis bags were centrifuged and the protein content of the supernatants estimated.

(d) *Electrophoresis*

Electrophoresis was carried out in a standard Tiselius apparatus (LKB) at 1°C for about 3 hr at 15 mA using protein solutions dialysed for at least 24 hr with stirring. Unless otherwise stated a "routine" buffer of pH 11.0 and ionic strength 0.1 and containing glycine (0.103M) and sodium hydroxide (0.1M) was employed.

(e) *Ultracentrifugation*

This was originally carried out on the Spinco model E ultracentrifuge at the Commonwealth Serum Laboratories, Parkville, through the courtesy of the Director, Dr. P. L. Bazeley, and Dr. J. O'Dea. The experiments were carried out at room temperature and the protein was dissolved in a buffer at pH 11.0 and ionic strength 0.3 (0.2M sodium chloride plus the glycine-sodium hydroxide buffer, ionic strength 0.1).

(f) *Analytical Data*

The determination of total sulphur in the various wool protein fractions was made in the C.S.I.R.O. Microanalytical Laboratory. Residual disulphide and mixed disulphide were estimated by the method of Leach (1959).

### III. RESULTS

(a) *Preliminary Electrophoretic Studies*

Moving-boundary electrophoresis was used to distinguish between the various proteins in the system. The initial experiments were designed to study the effect of *S*-carboxymethylation on the -SH protein and to determine the best conditions for maximum resolution.

Figure 1A shows the electrophoretic patterns of an unsubstituted -SH protein run at pH 11.0 in buffer (glycine 0.1M, thioglycolic acid 0.05M, KOH 0.2M, ionic strength 0.2), and Figure 1B the same protein after *S*-carboxymethylation and run in the routine buffer with the addition of 0.1M NaCl. It can be seen that both descending boundaries show similar numbers of components; this appears to be true also of the ascending boundaries, but evaluation is difficult in the latter because of the spike on the leading edge of the -SH protein which resembles those observed by Gillespie and Lennox (1955) in other wool protein solutions.

Figure 2 shows the influence of pH on the resolution of the wool proteins in 0.1 ionic strength buffers. It can be seen that resolution improved as the pH was increased to 11, fewer components being observed at lower pH values (cf. Woods 1959). At pH 12, although the ascending boundary showed better resolution, there was poor correspondence between the boundaries and during dialysis a portion of the protein precipitated from solution. In these figures at least three peaks are visible and in some experiments the large slow peak gave evidence of splitting; however, subsequent fractionation experiments have shown that the system was much more complex than would appear from the electrophoretic data.

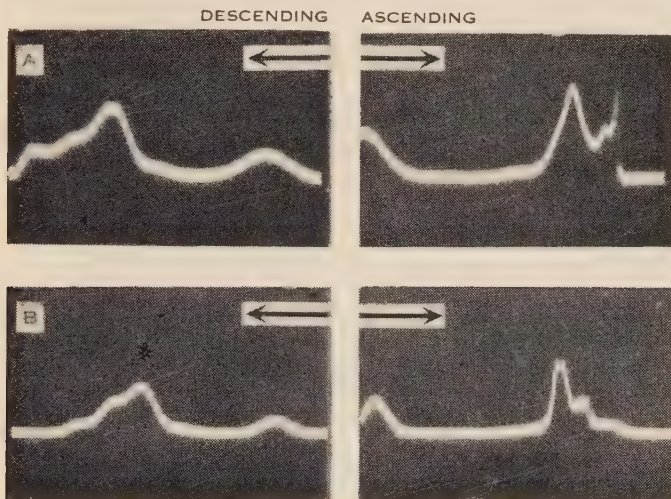


Fig. 1.—Effect of *S*-carboxymethylation on the electrophoretic pattern of the mixed wool proteins. *A*,  $-SH$  protein in the pH 11 buffer (glycine 0.1M, thioglycolic acid 0.05M, KOH 0.2M, ionic strength 0.2). Run for 360 min,  $2.6 \text{ V cm}^{-1}$ . *B*, *S*-carboxymethylated protein in the routine buffer with the addition of 0.1M NaCl. Run for 150 min,  $5.2 \text{ V cm}^{-1}$ .

Figure 3 shows, for comparative purposes, the pattern obtained by running at pH 11 the *S*-carboxymethyl derivative of the pooled protein from five successive pH 10.5 thioglycollate extracts of wool, made in accordance with the usual procedure employed in preparing keratine 2 (Gillespie and Lennox 1953). It can be seen that the pattern obtained was qualitatively comparable with that shown in Figure 2*B*, suggesting that the proteins obtained in the single-step extraction are similar electrophoretically to those obtained by the multi-step procedure but are obtained in differing proportions. However, they may differ in their content of  $-S-S-$  and bound thioglycolic acid (see Section IV).

#### (b) Ultracentrifuge Measurements

SCMK solutions were ultracentrifuged at 59,780 r.p.m. at protein concentrations of 1 per cent. The pattern obtained after 90 min running (Fig. 4) shows the presence of at least two peaks ( $S_{20w}$  1.3 and 2.9 respectively). In addition, the

presence of a small amount of rapidly sedimenting aggregated protein was indicated in earlier photographs.

(c) *Solubility Studies*

(i) *Acid Precipitation*.—The effect of pH on the solubility of SCMK was studied over the pH range 1–5 and the results are plotted in Figure 5. It can be seen that about three-quarters of the protein was precipitated sharply as the pH fell from

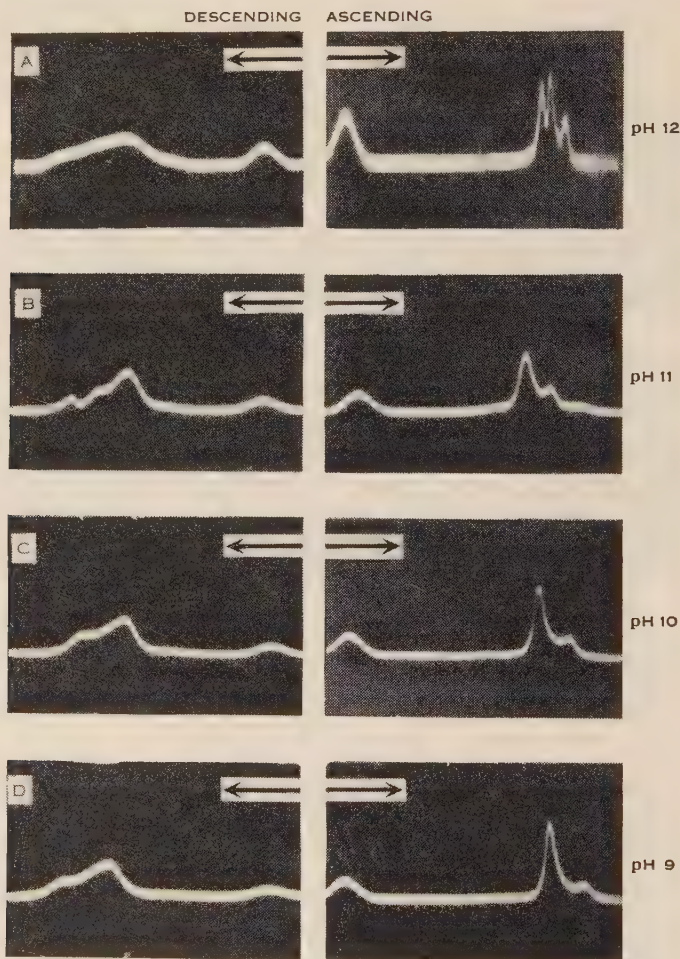


Fig. 2.—Effect of pH on the electrophoresis of SCMK in various buffers of ionic strength 0.1. *A*, pH 12, 135 min,  $5.2 \text{ V cm}^{-1}$ ; *B*, pH 11, 185 min,  $4.6 \text{ V cm}^{-1}$ ; *C*, pH 10, 180 min,  $5.4 \text{ V cm}^{-1}$ ; *D*, pH 9, 190 min,  $5.5 \text{ V cm}^{-1}$ .

4.7 to 4.5 and then the solubility decreased more gradually as the pH was lowered with the remainder of the protein showing a minimum at about pH 2.9. At lower pH values the solubility increased gradually.



(ii) *Salting Out*.—The solubility of SCMCK in ammonium sulphate was measured in the presence of sodium acetate buffer (ionic strength 0.1) at pH 6 (Fig. 6(a)). About two-thirds of the protein was precipitated sharply between zero and 0.4M concentration, then followed a zone of little additional precipitation, and finally almost all the remaining protein became insoluble between 1 and 2M ammonium sulphate concentration.

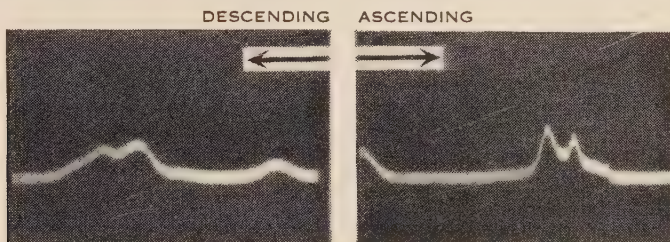


Fig. 3.—Electrophoresis pattern of the *S*-carboxymethyl derivative of the protein obtained by five successive pH 10.5 extracts of wool. Run in the routine buffer at pH 11 for 150 min, 5.3 V cm<sup>-1</sup>.

(iii) *Precipitation with Ethanol*.—The solubility of SCMCK was measured at pH 6 in sodium acetate buffer (ionic strength 0.01) at  $-5^{\circ}\text{C}$  with ethanol concentrations ranging from 15 to 70 per cent. The results presented in Figure 6(b) show that approximately two-thirds of the protein was precipitated as the ethanol concentration was increased from 30 to 40 per cent. Only small amounts of residual

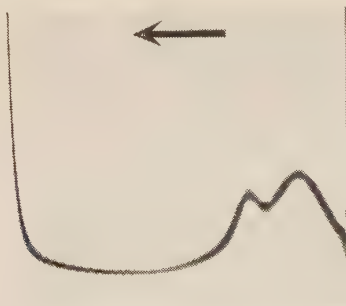


Fig. 4.—Ultracentrifuge pattern of SCMCK run at pH 11 in the routine buffer with the addition of 0.2M NaCl.

protein were precipitated even by 70 per cent. ethanol. After removal of ethanol by dialysis, the more easily precipitable protein gave highly turbid solutions at pH 7, whilst the residual proteins showed no turbidity under the same conditions. Sulphur analyses indicated that the precipitate obtained with 40 per cent. ethanol contained low-sulphur protein (2.8 per cent. S), whilst the more soluble proteins were rich in sulphur (6.3 per cent. S).



(iv) *Solubility in the Presence of Zinc.*—At pH 6 in the presence of sodium acetate buffer (ionic strength 0.01), 0.01M zinc acetate precipitated about two-thirds of the protein and the remainder was not precipitated even by increasing the zinc acetate concentration to 0.1M. This gave a very clean separation between the two types of proteins similar to that obtained during the purification of SCMKA2 (Gillespie 1957). But in view of the difficulties associated with the complete removal of bound zinc from the proteins (Gillespie and Springell 1957), this method was not used further.

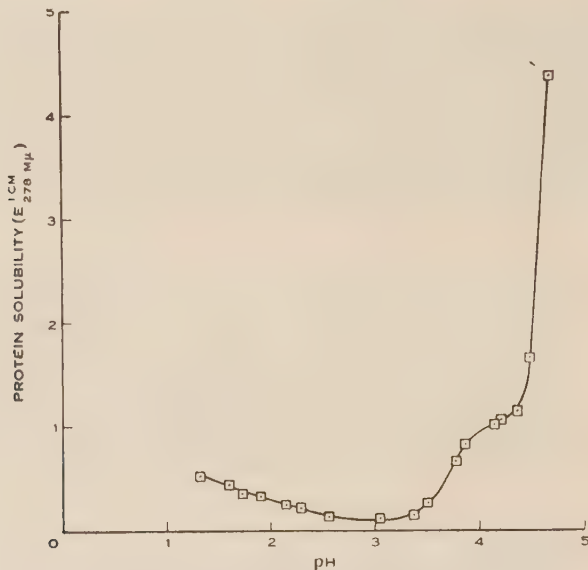


Fig. 5.—Solubility of SCMCK as a function of pH in phosphate-acetate buffers, ionic strength 0.2.

#### (d) *Separation of SCMCK into Two Main Fractions*

The most successful fractionation of SCMCK was obtained by acid precipitation. The procedure followed was to dialyse a 1 per cent. SCMCK solution against 10 volumes of 0.1M pH 4.1 acetate buffer (ionic strength 0.1) for 24 hr. The protein which precipitated formed a fibrous rope which had contracted from the walls of the dialysis tubing leaving an annulus of clear fluid. This precipitate was removed and a further quantity of the unprecipitated protein was obtained by washing it in a Waring Blendor with pH 4.1 acetate buffer and then filtering. These two lots of soluble unprecipitated protein, accounting for 30–35 per cent. of original SCMCK, were pooled, dialysed, and freeze-dried, and will be referred to as the “pH 4.1 supernatant fraction”. The precipitated protein was dissolved at pH 8, precipitated by dialysis, and the precipitate was washed. This procedure was repeated and all traces of the soluble fraction were thereby removed. Finally the “pH 4.1 precipitate fraction” was dissolved at pH 8, dialysed, and stored at  $-20^{\circ}\text{C}$ .

The electrophoretic composition of the original protein and of the two fractions was determined using the routine buffer (Fig. 7). The two fractions showed quite

different patterns; the precipitate fraction (Fig. 7*B*) had one peak containing about 60 per cent. of the material and two smaller peaks of faster material, whilst the supernatant fraction (Fig. 7*C*) was more heterogeneous and contained at least four or five components.

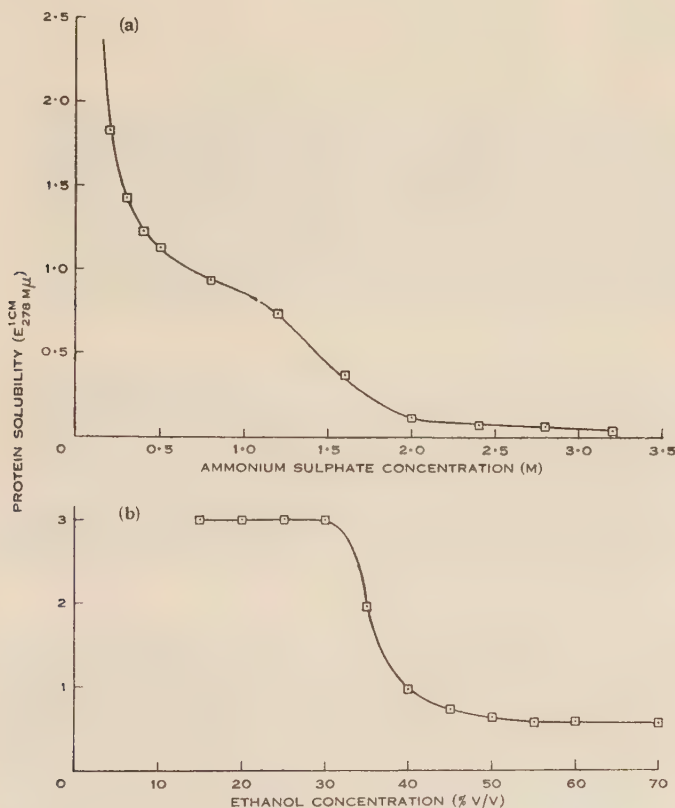


Fig. 6.—Solubility of SCM at pH 6: (a) as a function of ammonium sulphate concentration; (b) in acetate buffer of ionic strength 0.01 as a function of ethanol concentration.

Ultracentrifugation of the pH 4.1 precipitate fraction still showed two peaks (Fig. 8) but the amount of material in the slow-moving peak had been much reduced. The approximate sedimentation coefficients  $S_{20w}$  were now 1.9–2.2 and 2.9 respectively. It is known (Gillespie 1959) that the pH 4.1 supernatant fraction has an  $S_{20w}$  of 1.0 at a protein concentration of 1 per cent.

These differences were also reflected in the solubilities of the two fractions as measured by salting out and by acid precipitation. Figure 9 shows the pH-solubility relations of the two fractions and it can be seen that the two differ on this basis, the precipitate fraction precipitating sharply as the pH approaches 4.5 and then remaining almost completely insoluble at lower pH values, whilst the supernatant fraction showed a more normal type of solubility curve with a minimum around pH 2.9.

These differences between the fractions were also manifested in salting out experiments as illustrated by the curves in Figure 10. The precipitate fraction was easily salted out whilst the supernatant fraction required higher concentrations of ammonium sulphate. Other differences between these fractions were that the precipitate fraction was comparatively low in sulphur (2.7 per cent.), almost insoluble after freeze-drying, and gave solutions the turbidity of which increased with time.

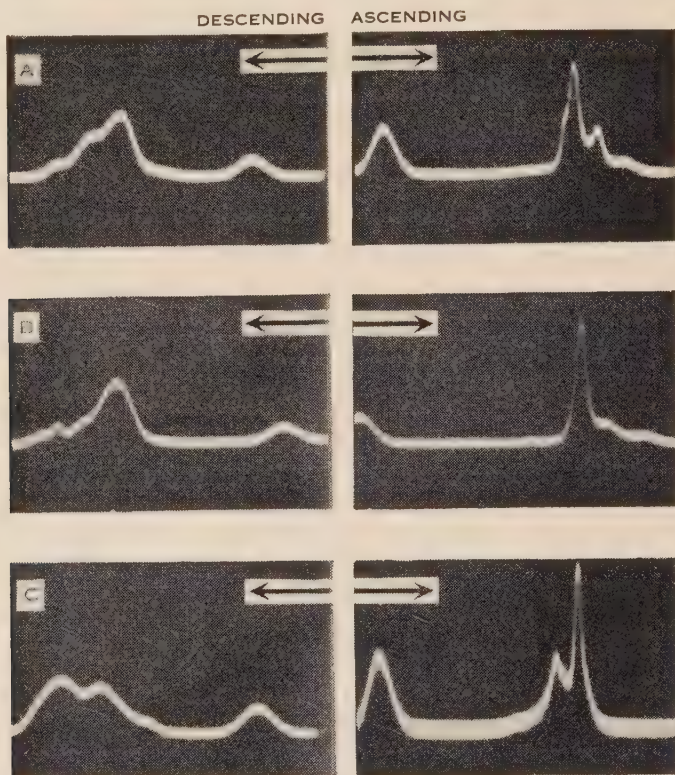


Fig. 7.—Fractionation of SCM by acid precipitation measured by electrophoresis in the routine buffer at pH 11: *A*, unfractionated SCM, 180 min,  $5.2 \text{ V cm}^{-1}$ ; *B*, precipitate fraction, 180 min,  $5.3 \text{ V cm}^{-1}$ ; *C*, supernatant fraction, 140 min,  $5.3 \text{ V cm}^{-1}$ .

On the other hand, the supernatant fraction contained a high concentration of sulphur (6.5 per cent.), gave permanently clear solutions suggesting little aggregation or small molecular weight, and was completely soluble after freeze-drying. A preliminary report on the separation of the proteins in this fraction has already been published (Gillespie 1959).

#### (e) *Purification of the pH 4.1 Precipitate Fraction*

The next stage of the fractionation procedures was designed to obtain the main peak protein in this fraction in an electrophoretically single-boundaried form. This

proved to be a difficult problem for the differences between the components were very small and they overlapped in most of their solubility relations.

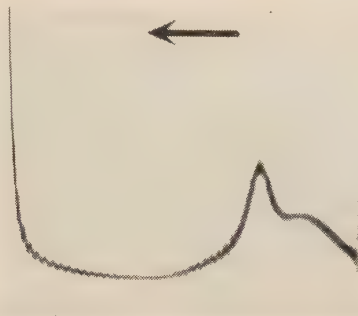


Fig. 8.—Ultracentrifuge pattern of the pH 4.1 precipitate fraction run at pH 11 in the routine buffer with the addition of 0.2M NaCl.

(i) *Salting Out*.—The proteins in this fraction were relatively easily precipitated at pH 6 even from solutions of univalent ions, and a study was undertaken of their

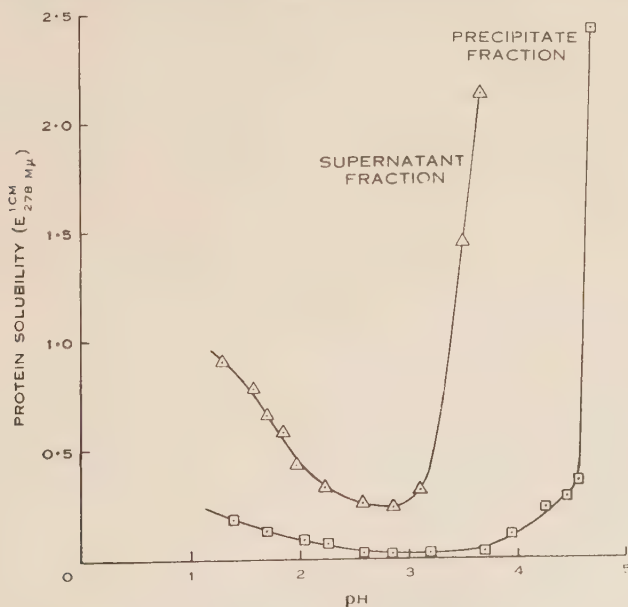


Fig. 9.—Solubility of the pH 4.1 supernatant and precipitate fractions of SCMK as a function of pH in phosphate-acetate buffers of ionic strength 0.2.

solubility in these solutions. Solubility curves at pH 6 (Fig. 11), using sodium chloride and sodium acetate-acetic acid as the precipitants, show that sodium chloride was less effective as a precipitant than ammonium sulphate (Fig. 10) and,



unexpectedly, that sodium acetate-acetic acid was more effective when compared on an ionic strength basis than sodium chloride and almost comparable with ammonium sulphate.

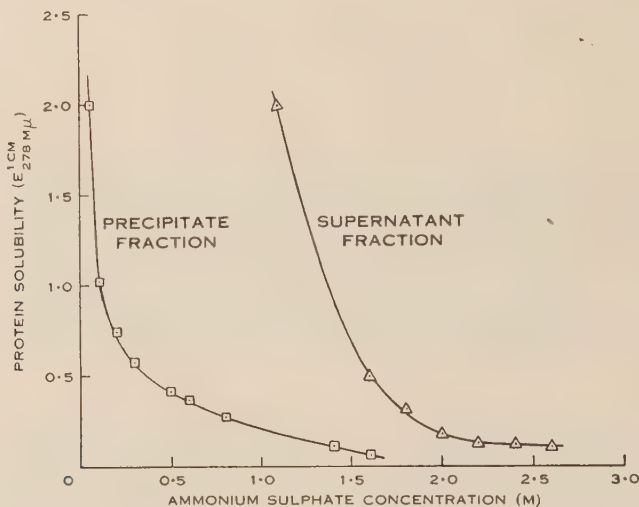


Fig. 10.—Solubility of the pH 4.1 supernatant and precipitate fractions of SCMCK as a function of ammonium sulphate concentration.

Both these solubility curves (Fig. 11) in salt solutions showed a sharp initial precipitation and then a levelling off at about 1.5M sodium chloride concentration

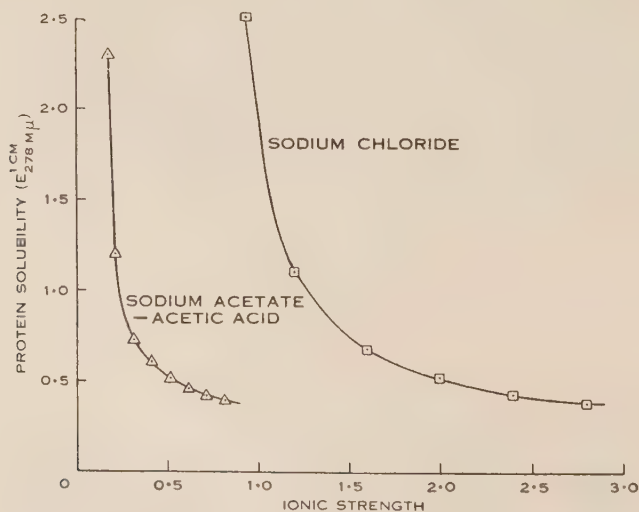


Fig. 11.—Solubility of the pH 4.1 precipitate fraction of SCMCK at pH 6 as a function of ionic strength.

and at an ionic strength of 0.3 for sodium acetate-acetic acid. Cuts were therefore made at these concentrations and also at a number of other concentrations on either

side, but electrophoretic analysis showed that only poor separations had been achieved. The best results were obtained at pH 6 with a 0.2 per cent. protein concentration and with a sodium acetate-acetic acid buffer concentration of ionic

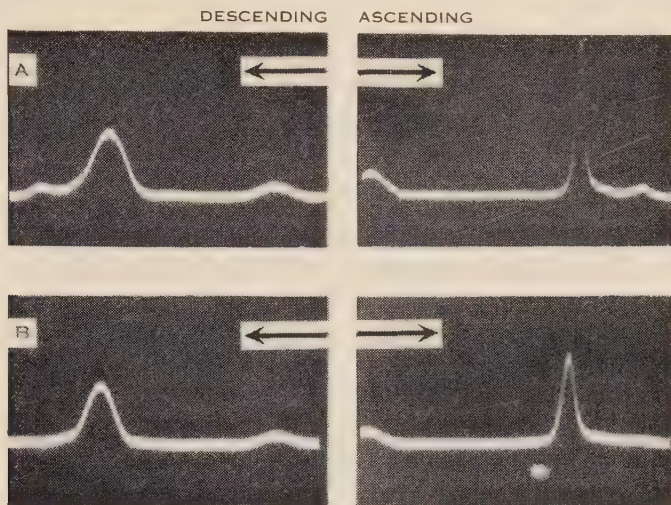


Fig. 12.—Fractionation of the pH 4.1 precipitate fraction measured by electrophoresis in the routine buffer at pH 11: *A*, salting out with sodium acetate-acetic acid buffer at pH 6, 180 min,  $5.2 \text{ V cm}^{-1}$ ; *B*, ethanol precipitation, 180 min,  $5.4 \text{ V cm}^{-1}$ .

strength 0.195. However, as Figure 12*A* shows, the major protein component was not pure.

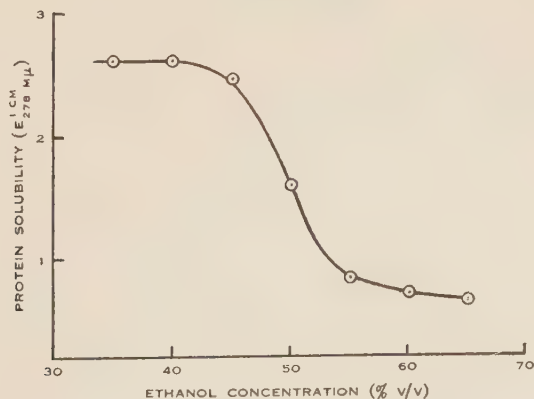


Fig. 13.—Solubility of the pH 4.1 precipitate fraction of SCM at pH 6 as a function of ethanol concentration.

(ii) *Precipitation with Ethanol*.—The low-temperature, low-ionic strength procedures of Cohn *et al.* (1946, 1950) were employed in the hope that their superior resolving power over salting-out procedures would enable the proteins to be sep-

arated. Precipitation by ethanol was measured at  $-5^{\circ}\text{C}$  in sodium acetate-acetic acid buffer at pH 6 and ionic strength 0.01 with a protein concentration of 0.2 per cent. From the results in Figure 13 it can be seen that most of the protein (80 per cent.) was precipitated over a very narrow range of ethanol concentration. Unfortunately the extent of precipitation was not reproducible; for example, in experiments with the same protein preparation under identical conditions of pH, temperature, ionic strength, protein concentration, rate of ethanol addition, and equilibrium time a certain degree of precipitation could be achieved with 50 per cent. ethanol

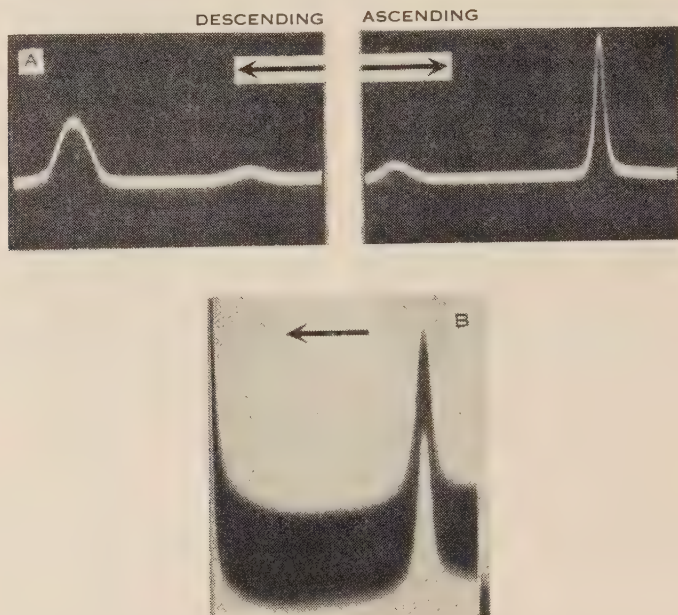


Fig. 14.—*A*, electrophoresis of SCMKA1 in the routine buffer at pH 11. Protein concentration 1.0 per cent., 180 min,  $5.4 \text{ V cm}^{-1}$ . *B*, ultracentrifugation of SCMKA1 at pH 11 in the routine buffer with added  $0.2\text{M}$  NaCl. Protein concentration 0.9 per cent.

on one day, whereas on the next day 40 or 60 per cent. ethanol may be necessary for the same result. There were no obvious reasons for this behaviour, but the appearance of the protein precipitates suggested that they might be coacervates, for they were very bulky and almost transparent. It was found very difficult to obtain partial precipitation of the protein using ethanol. However, when this was accomplished a fairly successful fractionation was obtained as shown by electrophoresis (Fig. 12*B*).

(iii) *Acetone Precipitation*.—The difficulties encountered when using ethanol were not experienced with acetone which gave normal precipitates and completely reproducible results from one experiment to another. The conditions of precipitation were the same as for ethanol. The protein was precipitated twice with an acetone concentration of about 50 per cent. v/v, the first arranged to precipitate about 65 per cent. of the protein and the second 90 per cent. The precipitated protein was dis-

solved and dialysed between precipitations. Because of the steepness of the precipitation curve it was found necessary to add the last 10 per cent. of acetone in several successive portions, estimating the protein concentration in the supernatant at the end of each addition in order to assess the extent of precipitation. For this type of comparative work the biuret test was used with no interval between mixing and reading. Under these conditions a fraction was obtained designated SCMKA1 which, when analysed electrophoretically showed only a single boundary (Fig. 14A). The mobility of this protein measured at a protein concentration of 1 per cent. in the routine buffer was  $7.7 \times 10^{-5} \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$  (cf. SCMKA2  $7.2 \times 10^{-5} \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$  (Woods 1959)).

When SCMKA1 was ultracentrifuged at 0.9 per cent. protein concentration in the routine buffer (pH 11) with the addition of 0.2M sodium chloride there was only one peak with a sedimentation coefficient of 2.5 (cf. SCMKA2 = 3.2 (O'Donnell and Woods 1956)) with no evidence of major impurity (Fig. 14B). However, the instrument used had a thick schlieren bar which gave a broad baseline and could possibly have obscured small amounts of impurities.

Although quite soluble at neutral pH, the solutions were very turbid and this opacity disappeared sharply as the pH was raised to between 10 and 11. It was at first thought that this might be due to the presence of a minor component of limited solubility because it could be centrifuged down in a Spinco model L centrifuge at 140,000 *g*. However, this is unlikely for, in a preliminary experiment at pH 7 to measure the size of the protein by light-scattering techniques at room temperature, the turbidity of a carefully clarified solution (centrifuged at 140,000 *g*) was observed to increase continuously with time; it had doubled in 2 hr and was visibly turbid after 18 hr. Providing the clarified solution was kept near or below its freezing point it remained clear for long periods.

This propensity for aggregation made physicochemical studies difficult. In electrophoresis experiments, for example, single peaks were obtained in both boundaries only at protein concentrations of about 1 per cent. or less. At higher concentrations, although the ascending limb retained a single peak, the descending limb showed the appearance of a hump, the size of which is increased in relation to the main peak with increasing protein concentration.

#### (f) *Amino Acid Composition of Purified Fractions*

Analysis of the pH 4.1 precipitate fraction and SCMKA1 by the ion-exchange column method (Table 2) reveal that the minor components removed by acetone fractionation must have a quite different amino acid composition from either SCMKA1 or SCMKA2, notably in having a much higher content of *S*-carboxymethyl cysteine (SCMC) and tyrosine. In spite of these and other differences they cannot be differentiated by acid precipitation and fall into the class of proteins precipitating at pH 4.1.

### IV. DISCUSSION

It is evident that SCMKA1 and SCMKA2 both fall into the class of proteins of lower sulphur content than whole wool and are possibly derived from the micro-



fibrils. There are many similarities between the two proteins, but there are differences, e.g. solubility, electrophoretic mobility, and sedimentation coefficient. It should be stressed that many of these properties are dependent on the extent of aggregation and possible chemical modification during the extraction procedures used. Physicochemical criteria of purity and homogeneity are difficult to apply to aggregating-disaggregating systems such as the wool proteins. It is not possible at present to establish whether these preparations are in fact distinct proteins or

TABLE 2  
AMINO ACID ANALYSIS OF WOOL FRACTIONS

Amino Acid	pH 4.1 Precipitate Fraction* ( $\mu$ moles/g)	SCMKA1 ( $\mu$ moles/g)	S. E.	SCMKA2† ( $\mu$ moles/g)	S. E.
<i>S</i> -Carboxymethyl cysteine	684	455	19.2	452	13.2
Aspartic	583	907	40.9	806	28.9
Threonine	446	471	27.7	415	19.2
Serine	795	801	40.9	721	28.9
Glutamic	888	1467	56.6	1243	39.7
Glycine	1075	702	33.7	600	24.1
Alanine	451	665	21.7	585	15.6
Valine	581	583	33.7	437	24.1
Isoleucine	228	336	13.2	330	9.6
Leucine	717	988	37.3	862	26.5
Tyrosine	538	393	20.5	313	14.4
Phenylalanine	288	249	21.7	248	15.6

\* This contains SCMKA1 and other proteins.

† Average results for several preparations.

slightly different forms of the one protein. Possible future lines of approach to this problem include the application of ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose using media favouring disaggregation. The most interesting differences are in the partial amino acid composition given in Table 2, which shows that in their contents of glutamic acid and tyrosine they differ significantly at the 1 per cent. level.

SCMKA1 contains some disulphide (0.14 per cent. S) probably partly as mixed disulphide and partly as cystine whereas SCMKA2 usually has none as measured by the procedure of Leach (1959) or by a qualitative nitroprusside test in the presence of cyanide. Table 3, taken from Human and Springell (1959), shows the contribution bound thioglycollate makes to this residual disulphide. The value of the re-resolution in thioglycollate step in reducing the amount of mixed disulphide is quite obvious and it can also be seen that SCMKA is comparable in this respect to the similar mixture of proteins produced by the five-step procedure.

At least 40 per cent. of the wool fibre is thus composed of proteins with a sulphur content less than half that of wool. As the 30–35 per cent. of wool left after exhaustive thioglycollate extraction has an amino acid composition not greatly different from that of wool itself (Gillespie and Simmonds, unpublished data), much of the wool sulphur must be concentrated in other fractions such as the pH 4.1 supernatant. If the thioglycollate extraction procedures remove pre-existing proteins from the fibre, then this observation is consistent with the existence of two histologically distinct classes of proteins in the wool fibre.

TABLE 3  
THIOGLYCOLLIC ACID CONTENT OF SCMKA PREPARATIONS

Method of SCMKA Preparation	Thioglycollic Acid Content ( $\mu$ moles/g protein)	S. E.
As prepared in this paper but without the steps involving acid precipitation followed by re-solution in alkaline thioglycollate	72.5 (mean of eight determinations)	5.6
As prepared in this paper	41 (mean of six determinations; lowest value 21)	6.5
Pooled five successive pH 10.5 extracts	33.0 (one determination)	—

It is of interest to compare the results presented here with those of Goddard and Michaelis (1934, 1935) who extracted wool with 0.5M sodium thioglycollate at pH 12 (initial pH) for 3 hr, obtaining about 60 per cent. of the wool in solution, and then coupled the dissolved proteins with iodoacetate. Their yield of protein was comparable with that obtained by Gillespie and Lennox (1955) using either one pH 12.6 extraction or a series of five successive pH 10.5 extractions followed by one of pH 12.2 (all initial pH values). Goddard and Michaelis thus had in their extracts, amongst others, the low-sulphur proteins SCMKA1 and SCMKA2 and the sulphur-rich proteins. They fractionated the extracts by salting out with ammonium sulphate from an 0.1M sodium acetate solution, probably at about pH 7.5, thereby obtaining two fractions: fraction A precipitating at 35 per cent. saturation (1.4M) and fraction B at 60 per cent. saturation (2.4M). Figure 15 shows a solubility curve in ammonium sulphate at pH 6 of the *S*-carboxymethyl derivative of a pH 12.6 (initial pH) thioglycollate extract of wool similar to that studied by Goddard and Michaelis. This shows points of inflexion near the ammonium sulphate concentrations used by these workers. Allowing for the greater solubility of the proteins at pH 7.5, their fraction A (3.3 per cent. S) would correspond to a mixture of SCMKA1 and SCMKA2 and other proteins precipitating at pH 4.1. All these proteins are characterized by virtual insolubility below the isoelectric point except under certain special conditions. Their fraction B (4.5 per cent. S) would correspond to the high-sulphur protein mentioned in this paper and to the zinc-soluble fraction obtained during the purification of

SCMKA2. The isoelectric point found for fraction B is very similar to the pH of minimum solubility (2.9) reported for the pH 4.1 supernatant fraction protein (Gillespie 1958). Fraction A contained more sulphur than  $\alpha$ -keratose (2.4 per cent.) (Alexander and Earland 1950) or SCMKA2 (1.5 per cent. as SCMC); in fact, fraction A had much the same content as the original wool. A possible explanation for these differences is that the "cystine S" recorded in their analysis is in fact mixed disulphide and that some sulphur is incorporated in an unknown form as found by Simmonds (1955), Gillespie (1958), and Human and Springell (1959). However, it should be noted in this connection that not all the pH 4.1 insoluble proteins are low in sulphur for, as Table 1 shows, the minor components removed from the pH 4.1 precipitate fraction during the acetone fractionation step were higher in sulphur than the purified protein,

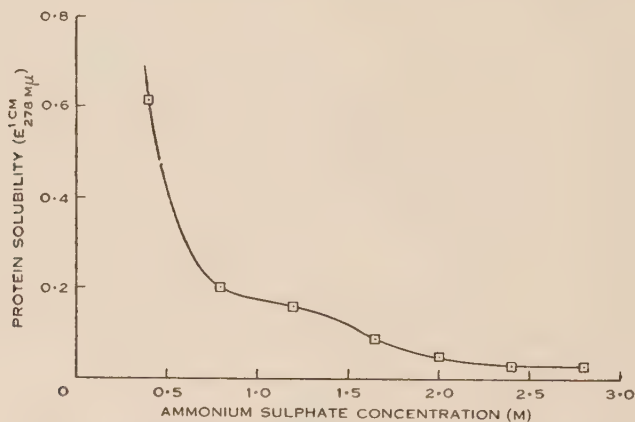


Fig. 15.—Solubility in ammonium sulphate at pH 6 of the *S*-carboxymethyl derivative of a pH 12.6 thioglycollate extract of wool.

the SCMC dropping from 684 to 450  $\mu$ moles/g protein during the separation. There appears to be a group of proteins precipitating at pH 4.1 but differing at least in sulphur content. Their fraction B contains a higher concentration of sulphur than does wool, but not as high a concentration as the pH 4.1 supernatant fraction obtained in the present study nor as high as  $\gamma$ -keratose (6.1 per cent.) and it seems likely that the differences found in this Laboratory between the pH 4.1 precipitate fraction and fraction A on the one hand and between the pH 4.1 supernatant fraction and fraction B on the other lie in the imperfect condition obtained by ammonium sulphate salting out as compared with isoelectric precipitation.

In some preparations of SCMKA1 some sulphur is unaccounted for on the basis of the content of sulphur-containing amino acids. This has been previously observed by Fraenkel-Conrat (1942) in experiments on the reduction by thioglycollate of the lactogenic hormone and by Simmonds (1955) in his analyses of wool kerateine. Part of this sulphur is probably mixed disulphide due to incomplete reduction of the protein or to partial oxidation in later handling, as shown by Springell (1958) and Human and Springell (1959) using  $^{35}\text{S}$ -thioglycollate. The remainder may come from a contaminant in the thioglycollate, or be produced from it during the extraction,



which combines chemically with the wool. The work of Schoberl (1948) and White (1959) showing that thioesters contaminating thioglycollic acid can condense with the  $\epsilon$ -amino groups of lysine residues in proteins to introduce new  $-SH$  groups is very relevant to this problem and may in fact provide the explanation. All these results serve to emphasize the desirability of carefully purifying thioglycollic acid before using it for the reduction of proteins.

The interpretation of these facts, in the light of knowledge concerning the bilateral structure of wool now available, is difficult. Fraser and Rogers (1953) originally considered that the first fractions of protein extracted from wool by alkaline thioglycollate had their origin in the ortho segment whilst the more difficultly extractable material, e.g. SCMKA2, originated in the para region of the fibre. However, Alexander and Smith (1956) in discussing this problem considered that, in so far as their peracetic-ammonia process was concerned, the difference between the two segments lay in their different proportions of fibrillar and matrix protein rather than in having distinct types of proteins. The recent work of Rogers (1959b) would seem to confirm this view.

If the view of these workers is correct it seems difficult to explain the observations we have made on the partial extraction of wool proteins with thioglycollate (Gillespie and Lennox 1955). When wool was extracted with pH 10.5 thioglycollate several extractions were required for the pH to reach 10.5 and there was an increase in the amount of protein extracted in the first two extractions but thereafter the amount in each succeeding extract diminished and after the fifth virtually no more protein came out of the fibre, even though almost all the cystine had been reduced by the end of the third extraction. However, if the pH was raised to about 11.4, further protein could be removed in a series of extractions until a limit was reached again. In order to extract further quantities of protein it was necessary to raise the pH once more or to add a reagent such as urea. Harrap (unpublished data) found that if wool was treated once with 0.5M thioglycollate at pH 10.5, all the thioglycollate-extractable material could be obtained in one operation by washing the fibres with a large volume of distilled water. Microscopic examination showed that the fibres and membranes were disrupted, probably by osmotic action thereby allowing the proteins to escape. This suggests that an important step in the extraction process may be membrane rupture. If SCMKA1 and SCMKA2 are not in different locations in the fibre then they may be contained in membranes of differing degrees of resistance to alkali. As some high-sulphur protein is released with both SCMKA1 and SCMKA2 this implies that both microfibril and matrix are enclosed in these membranes of varying strength. Mercer (1953) suggested that the bulk of the protein occurred as "small packets", each enclosed in a non-keratinous cortical cell membrane which was resistant to reduction but was susceptible to attack by alkaline solutions. Whilst intact or unswollen these membranes would certainly restrict the passage of proteins and if there is a gradation in resistance to alkali, then this could explain our observations.

There is an alternative interpretation of the extraction phenomena: that the matrix-microfibrillar protein complex occurs in different states of molecular organization or different degrees of packing. It is known that only a portion of the wool



fibre is crystalline, estimates varying from 10 per cent. to a maximum of something less than 50 per cent. (Alexander and Hudson 1954; Fraser and MacRae 1957*a*, 1958). Alexander and Hudson suggest that by analogy with the synthetic fibres, the individual micelles of the wool fibre also contain crystalline regions. Presumably this could affect the rate of extraction and the pH and time required to produce a necessary degree of swelling. The well-known differing rates of solubility between the amorphous and crystalline forms of the same protein illustrate the important influence of these two states. The only evidence supporting this view is the observation by Fraser and MacRae (1957*b*) that the residue remaining after wool had been extracted with alkaline thioglycollate under conditions which removed only part of the soluble proteins had a higher density and, presumably, higher crystallinity than the original fibre. These workers point out, however, that the interpretation of their density measurements on partly extracted wools may be open to some question because of possible variations in accessibility to the molecules of the immersion liquid. Yet the results as they stand suggest that the more easily extractable proteins originate in the less crystalline regions of the fibre.

It has been observed in this connection (Gillespie and Simmonds, unpublished data) that the matrix proteins in the native state are basic and this basicity increases with difficulty of extraction, whilst the accompanying microfibrillar proteins are slightly acidic. The matrix-microfibrillar complex isolated at pH 10.5 must be less basic than the material isolated at pH 11.4, and this implies that this latter complex requires a higher net charge by discharge of basic groups before it can be broken into its constituent proteins.

The work of Ryder (1956, 1958), which shows that a considerable proportion of the sulphur in the fully formed wool or hair fibre is introduced at a point in the follicle immediately above the bulb, suggests that the high- and low-sulphur proteins may be produced at separate times and sites and that they may have no covalent bonding between them. This is supported by the work of Lindley (1947) and Blackburn (1959) who were able to separate high- and low-sulphur proteins by methods not involving disulphide bond fission.

With the recognition that the widely used peracetic and performic oxidative methods of disulphide bond rupture also modify proteins in other ways (Corfield, Robson, and Skinner 1958; Moore *et al.* 1958) the study of the alternative method of reduction followed by alkylation has received a new impetus. The role played by thioglycollic acid in introducing new -SH groups into proteins has already been discussed. In addition, however, Michaelis and Schubert (1934) have shown that it is possible for iodoacetate to carboxymethylate the  $\epsilon$ -amino groups of lysine residues and this reaction has been studied in detail using bromoacetate (Korman and Clarke 1956). The latter workers and Moore *et al.* (1958), and Gundlach, Stein, and Moore (1959) have also shown that iodoacetate can also react with tyrosine and histidine residues. Examination of amino acid analyses made in this Laboratory on soluble proteins from wool such as SCMKA1 and SCMKA2 has failed to reveal the presence of any of the expected modified amino acids. It may well be that our reaction conditions, employing as they do temperatures below 20°C and pH values below 9 for the alkylation reaction followed by dialysis at pH 7, do not favour the

formation of these reaction products. Alternatively, the appropriate residues of wool may be less reactive than those in other proteins.

## V. ACKNOWLEDGMENTS

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## APPENDIX I

### SUMMARY OF NOMENCLATURE OF PROTEINS EXTRACTED FROM WOOL

It seems desirable at this stage to simplify and clarify the nomenclature of proteins extracted from wool by reduction and subsequent protection of the liberated -SH groups, for example by alkylation. The method used in earlier papers from this Laboratory of numbering on the basis of electrophoretic mobility was sufficient for a simple system but the growing complexity now evident and the isolation of proteins with similar mobilities necessitates some revision of this method. Alexander and Earland (1950) made use of a preliminary subdivision of proteins extracted from oxidized wool on the basis of sulphur content and termed these respectively  $\alpha$ - and  $\gamma$ -keratoses. Some years previously Goddard and Michaelis (1935) had named their low-sulphur protein fraction "A" and the high-sulphur fraction "B". In view of the now evident histological localization of these two types of protein, this system would appear to have a good deal of merit. It is proposed therefore to convert to this system, so that all reduced proteins with a sulphur content equal or lower than wool will be termed "kerateine A" and those higher than wool "kerateine B", with the corresponding derivatives, in the case of alkylation with iodoacetic acid, *S*-carboxymethyl kerateine A and B respectively. Then in an arbitrary fashion the individual proteins can be labelled by inserting a number after the letter.

Using this revised nomenclature it is proposed that *S*-carboxymethyl kerateine 2 (SCMK2) be now called *S*-carboxymethyl kerateine A2 (SCMKA2) and the readily extractable low-sulphur protein described in this paper *S*-carboxymethyl kerateine A1 (SCMKA1).

Table 4 summarizes the present knowledge of fractionation of the reduced wool proteins.

TABLE 4

FRACTIONATION OF WOOL (MERINO 64's) BY FRACTIONAL EXTRACTION WITH ALKALINE  
THIOGLYCOLLATE FOLLOWED BY REACTION WITH IODOACETATE

Multiple-extraction Method	Single-extraction Method
<p>Successive extractions with 0.1M thioglycollate at 50°C for 20 min. Liquor: wool ratio 30 : 1. 65-70 per cent. of wool dissolves</p>	<p>One extraction at pH 10.5 for 2 hr at 50°C. Liquor: wool ratio 100 : 1. 35-40 per cent. of wool dissolves</p>
<div style="display: flex; align-items: center; justify-content: center;"> <div style="text-align: right; margin-right: 10px;">           Final pH 10.0 10.3 10.5 10.5 11.4         </div> <div style="font-size: 3em; margin-right: 10px;">}</div> <div>Discard</div> </div>	<p>Cool, precipitate at pH 5. Redissolve in thioglycollate at pH 10</p>
<p>Cool, react with iodoacetate pH 8.5-9. Dialyse at pH 6-7</p>	<p>React with iodoacetate pH 8.5-9. Dialyse at pH 6-7</p>
<p>Fractionate with zinc acetate</p>	<p>Fractionate at pH 4.1</p>
<div style="display: flex; justify-content: space-around;"> <div style="text-align: center;"> <p>Precipitate</p> <p><i>S</i>-carboxymethyl keratine A2 (SCMKA2)</p> </div> <div style="text-align: center;"> <p>Supernatant</p> <p>High-sulphur proteins</p> </div> </div>	<div style="display: flex; justify-content: space-around;"> <div style="text-align: center;"> <p>Precipitate</p> <p>4 low-sulphur proteins</p> <p>Fractionate with acetone</p> <p>Precipitate</p> <p><i>S</i>-carboxymethyl keratine A1 (SCMKA1)</p> </div> <div style="text-align: center;"> <p>Supernatant</p> <p>4 or 5 high-sulphur proteins</p> <p>Supernatant</p> <p>3 low-sulphur proteins</p> </div> </div>



## SHORT COMMUNICATIONS

### NITRATE ACCUMULATION IN RELATION TO MOLYBDENUM DEFICIENCY LEAF SCORCH SYMPTOMS\*

By L. F. NOTLEY† and G. L. WILSON‡

The accumulation of nitrate in the leaf tissue of nitrate-supplied, molybdenum-deficient plants has been suspected as a cause of the scorching symptoms which frequently develop (e.g. Agarwala 1952; Agarwala and Hewitt 1952; Hewitt and McCready 1953). Quantitative studies of the relationship between nitrate content and the symptoms have, however, been inconclusive. The literature suggests (cf. Wilson and Waring 1948; Johnson, Pearson, and Stout 1952) that this may follow from the estimation of nitrate in whole leaf tissue rather than in damaged portions.

Molybdenum-deficient lettuce and tomatoes were available from other work (Wilson and Notley 1959) and some observations were made on nitrate contents and distribution in the leaf in relation to the scorch symptoms.

#### *Methods and Results*

Two series of determinations were made on lettuce and one on tomatoes, as scorch symptoms were available from plants of varying ages. Damaged tissue was removed as soon as the water-soaked appearance (which precedes the rapid drying out to give the papery scorch symptoms) was observed, and nitrate determined by a modified form of Horne and Denmead's (1955) brucine test. Content is expressed on a fresh weight basis and Table 1 shows the ranges of values obtained in these three series.

An experiment was designed to compare the nitrate contents in (1) injured interveinal areas; (2) uninjured interveinal areas; and (3) uninjured areas containing portion of a large vein. These last two classes of material were also taken from molybdenum-supplied control plants. Six replicates of material, on each of two occasions, were taken from each species. Mean nitrate contents are shown in Table 2, while the range of individual observations in the case of injured and uninjured interveinal areas from molybdenum-deficient plants is shown in Table 1 as series 4.

#### *Discussion*

The minimal nitrate contents in damaged tissue are close within each species: 2.2–2.9 per cent. in lettuce and 2.8–3.9 per cent. in tomatoes. Maxima in undamaged tissue are in close agreement with these minima for both species. Minima in undamaged tissue are of no significance here, merely representing earlier stages of accumulation. The high maxima in damaged tissue can be attributed to delay in taking samples from tissue which is rapidly drying out.

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TABLE 1

RANGE OF NITRATE CONTENT IN INJURED (SCORCHED) AND UNINJURED PORTIONS OF LAMINA FROM MOLYBDENUM-DEFICIENT LETTUCE AND TOMATO PLANTS

Plant	Series	No. of Observations	Nitrate Content (per cent. fresh wt.)	
			Uninjured	Injured
Lettuce	1	40		2.5 - 5.0
	2	44		2.2 - 6.2
	4 (1st date)	6	1.1 - 2.6	2.9 - 3.8
	4 (2nd date)	6	1.5 - 2.3	2.2 - 3.5
Tomato	3	50		2.8 - 5.5
	4 (1st date)	6	1.5 - 2.8	3.9 - 5.4
	4 (2nd date)	6	1.6 - 3.0	3.0 - 4.2

TABLE 2

COMPARISON OF MEAN NITRATE CONTENTS (PER CENT. FRESH WEIGHT) IN VARIOUS PORTIONS OF LEAVES FROM MOLYBDENUM-DEFICIENT AND CONTROL LETTUCE AND TOMATO PLANTS

Plant	Sampling Date	Molybdenum-deficient Plants			Least Significant Difference ( $P = 0.05$ )	Control Plants*	
		Injured Lamina	Uninjured Lamina	Uninjured Lamina with Large Vein		Lamina Only	Lamina with Large Vein
Lettuce	6.viii.56	3.26	1.92	1.06	0.51	0.0	0.5
	13.viii.56	3.19	1.55	1.15		0.0	0.5
Tomato	7.iii.56	4.61	2.26	1.20	0.70	0.0	0.6
	18.iv.56	3.72	2.48	1.02		0.0	0.6

\* Not included in analyses.

The regular association between symptom appearance and nitrate level suggests, but does not prove, a cause and effect relationship. Scorch symptoms characterize a number of mineral deficiencies but some 3 per cent. nitrate on a fresh weight basis might well be toxic. The actual distribution of this ion may represent much higher concentrations again at certain sites.

The pattern of nitrate distribution in the leaves of molybdenum-supplied and molybdenum-deficient plants clearly indicates the inadequacy of whole leaf determinations in seeking relationships between nitrate contents and the appearance of symptoms in localized parts of leaves.

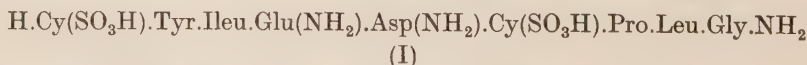
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## SELECTIVE DEGRADATION BY BROMINE WATER OF THE POLYPEPTIDE CHAINS OF OXIDIZED INSULIN\*

By E. O. P. THOMPSON†

In degradative work on oxytocin (Mueller, Pierce, and du Vigneaud 1953; Ressler, Trippett, and du Vigneaud 1953; du Vigneaud *et al.* 1954) it was found that whereas oxidation with performic acid gave a single product (I), oxidation with



bromine water or treatment of (I) with bromine water gave two peptide fragments, resulting from cleavage of a dibromotyrosylisoleucyl bond. The reaction did not depend on the isoleucyl residue, since in vasopressin, where the sequence is -tyrosyl-phenylalanyl-, a similar cleavage with bromine water was noted (Popenoe and du

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Vigneaud 1953). This fragmentation is one of the few known selective degradations (Thompson 1959b) by a chemical method and it was of interest to apply the method to other polypeptides to determine whether the reaction was general.

### Experimental

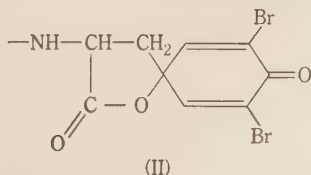
The glycy chain of oxidized insulin contains a  $-\text{Tyr.Cy}(\text{SO}_3\text{H}).\text{Asp}(\text{NH}_2).\text{OH}$  sequence (Sanger and Thompson 1953) and treatment of this polypeptide with bromine water in aqueous methanol (4:1 v/v) at  $-5$  to  $-10^\circ\text{C}$  gave rise to the peptide  $\text{H.Cy}(\text{SO}_3\text{H}).\text{Asp}(\text{NH}_2).\text{OH}$  which was readily isolated by paper chromatography (Sanger and Thompson 1953). To obtain a quantitative estimate of the extent of the cleavage the reaction mixture was dinitrophenylated (Sanger 1945) and after acid hydrolysis the dinitrophenyl (DNP)-amino acids were separated and estimated. The ether extract of the hydrolysate contained DNP-glycine, arising from the original *N*-terminal residue, and DNP-glutamic acid which were separated by paper chromatography and estimated by the method of Levy (1954). The aqueous phase contained DNP-cysteic acid which was estimated after separation by paper ionophoresis (Thompson 1959a). The yields of DNP-glutamic acid and DNP-cysteic acid varied from 17 to 32 per cent. and 34 to 42 per cent., respectively, of the amount of DNP-glycine after applying a correction (Porter and Sanger 1948) for the destruction of DNP-amino acids during hydrolysis.

The treatment with bromine water (1 hr,  $-5$  to  $-10^\circ\text{C}$ ) was next applied to the phenylalanyl chain of oxidized insulin followed by dinitrophenylation, hydrolysis, and separation and estimation of the DNP-amino acids. In addition to the original phenylalanyl *N*-terminal residue, DNP-derivatives detected were those of threonine and leucine in yields of 13 per cent. and 19 per cent., respectively, of the DNP-phenylalanine.

With both these polypeptides the new *N*-terminal residue corresponded with those which are linked by their amino groups to tyrosyl groups, thus demonstrating the wide specificity of the reaction. The yields obtained in these experiments were much lower than those obtained during selective degradation by enzymes, but reaction conditions for the glycy chain of oxidized insulin were varied only between periods of 1 hr at  $-5$  to  $-10^\circ\text{C}$  followed by acid treatments ranging from 0.01N to 1N HCl at  $-5^\circ\text{C}$  or room temperature for 1 hr; or treatment with 0.1N ammonia for 1 hr at room temperature.

### Discussion

Further work on this method has recently been published by Corey and Haefele (1959) and Schmir, Cohen, and Witkop (1959). These workers have elucidated the course of the reaction, which involves an oxidation during which the phenolic ring of the tyrosyl residue is converted to a dienone-lactone structure (II) with cleavage





of the dibromotyrosyl peptide bond. Near quantitative yields were obtained with simple model compounds while *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucine was found by Schmir, Cohen, and Witkop (1959) to release 40 per cent. of isoleucine after oxidative bromination.

Low yields in this selective oxidation may limit the usefulness of this reaction in protein chemistry to the rapid determination of residues linked to the carboxyl group of tyrosine. Bromine oxidation of cystine residues in proteins such as insulin and papain has previously been found to be non-quantitative (Thompson 1956) but the possibility that denaturation by agents such as urea or detergent would increase the extent of reaction, as is the case during reduction (Thompson 1959*b*), was not investigated.

For proteins containing tryptophan Patchornik, Lawson, and Witkop (1958) have shown that tryptophyl peptide bonds are also readily cleaved by the action of bromine or *N*-bromosuccinimide but Ramachandran and Witkop (1959) have found conditions where tryptophyl bonds are attacked without affecting tyrosyl peptide bonds. Ramachandran and Witkop (1959) found that tryptophyl bond cleavages in proteins treated with *N*-bromosuccinimide in the presence of urea only averaged 20–40 per cent. and were often very low.

#### *Acknowledgment*

The author wishes to thank Dr. S. J. Leach for samples of the chains of oxidized insulin purified by countercurrent distribution.

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# THE RESPIRATORY CHAIN OF BEETROOT MITOCHONDRIA

By J. T. WISKICH,\* R. K. MORTON,† and R. N. ROBERTSON\*

[Manuscript received November 9, 1959]

## Summary

Mitochondria were isolated from root tissue of red beetroot (*Beta vulgaris* L.) and the components of the respiratory chain for oxidation of succinate and of reduced diphosphopyridine nucleotide (DPNH) were studied. Succinate, DPNH, ferrocytochrome *c*, and malate were used as substrates, and 2,6-dichlorophenol-indophenol, ferricytochrome *c*, and oxygen as hydrogen (electron) acceptors. DPNH was oxidized without addition of cytochrome *c* and malate without addition of DPN. These observations suggest that the respiratory chain was retained intact in the isolated mitochondria. Cytochromes *b*, *c*<sub>1</sub>, and *c* were identified spectroscopically by the positions of their characteristic  $\alpha$ -absorption bands. The very small amount of cytochrome *c* present may indicate some loss of this component during isolation of the mitochondria. An absorption band near 600  $\mu$  was attributed to cytochromes (*a*+*a*<sub>3</sub>).

Succinate-cytochrome *c* reductase was strongly inhibited by antimycin A and somewhat less by 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO), 2-hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone (SN 5949), and phenylurethane. These results suggest that there is only one pathway for hydrogen transport between succinate and cytochrome *c*. In contrast, the DPNH-cytochrome *c* reductase was incompletely inhibited by antimycin A, HOQNO, and SN 5949. Amytal strongly inhibited this system. However, antimycin A and amytal were less inhibitory when tested with the DPN-stimulated oxidation of malate. The results suggest that these are alternative pathways for oxidation of DPNH.

The rates of oxidation of succinate, DPNH, and of ferrocytochrome *c* were all stimulated by freezing and thawing or by hypotonic treatment of the freshly isolated mitochondria. With malate and with succinate as substrates, P/O ratios of 0.8 and 2.0 respectively were obtained. The results have been compared with those obtained with mitochondria from animal and other plant tissue.

## I. INTRODUCTION

It has been shown that mitochondria, isolated from a wide variety of plant and animal tissues, are the site of aerobic respiration and that there is a definite sequence of hydrogen and electron carriers involved in the oxidation of substrates (see Chance and Williams 1956). Although studies on plant mitochondria have been less extensive, it appears that a number of the respiratory components are similar to those of animal mitochondria. However, it cannot be assumed that the respiratory pathways in plant and animal mitochondria are identical.

Known properties of cytochrome components of plant tissues have been reviewed by Hill and Hartree (1953), Hartree (1957), Morton (1958), and Smith

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and Chance (1958). The latter workers have drawn attention to some of the conflicting observations by different workers.

Martin and Morton (1956*a*, 1956*b*, 1957) carried out enzymic, spectroscopic, and spectrophotometric studies to determine the functions and properties of cytochromes of mitochondria and microsomes isolated from silver-beet petioles and from wheat roots. Spectrophotometric observations of the cytochromes of particles from wheat roots by Lundegårdh (1958) are in general agreement with the results of Martin and Morton (1957) except for the localization of some of the pigments. Particles from cauliflower buds (Crane 1957) and tobacco roots (Sisler and Evans 1959) have been used for similar studies.

Honda, Robertson, and Gregory (1958) studied the effect of salts on the oxidation of reduced diphosphopyridine nucleotide and of reduced cytochrome *c* by beetroot mitochondria, but did not determine the components of the oxidation systems. This paper describes studies to determine the sequence of reactions in the respiratory chain of beetroot mitochondria.

## II. MATERIALS AND METHODS

### (a) *Preparation of Mitochondrial Suspension*

The mitochondria were isolated from red beet (*Beta vulgaris* L.) obtained commercially. The tissue (200 g), diced into cubes of approx. 0.5 cm, was blended for 30 sec in a medium (220 ml) of 0.4M sucrose, containing 0.045M tris(hydroxymethyl)aminomethane (TRIS) and 0.005M ethylenediaminetetra-acetic acid (EDTA). The homogenate was strained through a double layer of muslin and centrifuged at 1500 *g* for 10 min. The supernatant was again strained and then centrifuged at 6000 *g* for 30 min. The precipitate was suspended in 0.4M sucrose and centrifuged at 20,000 *g*. The mitochondria were washed at least once to remove the red pigment and the final suspension was made up to a volume of 10 ml. All operations were carried out in the cold (about 1°C) with chilled solutions and apparatus. Centrifuging was carried out at -3°C. The suspension was kept in an ice-bath during the course of an experiment.

### (b) *Measurement of Enzymic Activities*

The general procedures have been described by Martin and Morton (1956*a*). A Beckman spectrophotometer, model DU, with photomultiplier attachment was used in a room maintained at 25°C. All reaction mixtures were made up to a final volume of 3 ml in cuvettes with 1 cm light path. The reactions were started by the addition of the mitochondrial suspension and reaction rates were estimated from the changes in optical density (O.D.) at the appropriate wavelengths. Molar extinction coefficients of  $6.22 \times 10^3$  at 340 m $\mu$  for reduced diphosphopyridine nucleotide, of  $19.6 \times 10^3$  (reduced minus oxidized compound) at 550 m $\mu$  for cytochrome *c*, and  $19.1 \times 10^3$  at 600 m $\mu$  for 2,6-dichlorophenolindophenol, were used for estimation of these compounds.

The reaction mixtures contained 31 mM TRIS-28 mM acetic acid buffer, at pH 7.2. Where used, inhibitors were added to the reaction mixture before addition of enzyme.

All activities are expressed as the change in optical density ( $\Delta$  O.D.)/hr/mg mitochondrial nitrogen, as estimated at the appropriate wavelength for the first 30 sec of reaction.

Oxidative phosphorylation was determined essentially as described by Hunter (1955) with air as the gas phase. The manometer vessel contained 0.27M sucrose, 31 mM TRIS, 28 mM acetic acid, 2  $\mu$ M cytochrome *c*, 13 mM potassium phosphate, 1 mM adenosine diphosphate, 6.7 mM  $\text{MgCl}_2$ , 10 mM NaF, 20 mM glucose, approx. 1 mg of hexokinase, and 370  $\mu$ g mitochondrial nitrogen per reaction mixture (3 ml). Oxygen uptake was measured at 25°C for about 30 min. The reaction was then stopped with 5 per cent. perchloric acid and glucose 6-phosphate formed enzymically was estimated spectrophotometrically with glucose 6-phosphate dehydrogenase and triphosphopyridine nucleotide, after neutralization.

Attempts to estimate oxidative phosphorylation with DPNH by direct spectrophotometry according to Pullman and Racker (1956) were unsuccessful because of the high adenylate kinase activity. About 50 per cent. inhibition of this adenylate kinase activity was obtained with sodium fluoride (0.01M).

#### (c) *Estimation of Nitrogen*

Samples were digested in concentrated sulphuric acid with mercury as catalyst. Distillations and titrations were carried out essentially as described by McKenzie and Wallace (1954).

#### (d) *Chemicals*

Diphosphopyridine nucleotide (DPN) and reduced diphosphopyridine nucleotide (DPNH) were obtained from C. F. Boehringer and Soehne, Mannheim, Germany. Adenosine diphosphate (ADP) and triphosphopyridine nucleotide (TPN) were obtained from Pabst Laboratories, Wisconsin, U.S.A. Hexokinase and glucose 6-phosphate dehydrogenase were supplied by Sigma Chemical Co., St. Louis, U.S.A. Cytochrome *c* was prepared according to the method of Keilin and Hartree as modified by Potter (1951), and then dialysed against distilled water. Reduced cytochrome *c* was prepared by reduction with sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ), the excess dithionite being oxidized with a stream of air.

Amytal (5-ethyl-5-isoamylbarbiturate, sodium salt) was obtained from Eli Lilly and Co. Ltd. Other inhibitors were gifts from various workers. 2-Hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone (SN 5949) and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) were made up in 1 per cent. NaOH, and antimycin A and phenylurethane in 40 per cent. (v/v) ethanol.

### III. RESULTS

Some of the factors affecting the rates of aerobic oxidation of both DPNH and of reduced cytochrome *c* by beet mitochondria have been described by Honda, Robertson, and Gregory (1958). In the present work a set of conditions was chosen and used for all enzymic reactions. Seasonal variations in enzymic activity occurred (cf. Martin and Morton 1956*a*) but the rate of aerobic oxidation of DPNH was used as a reference for comparison of different preparations.



*(a) DPNH Oxidase Activity*

The rate of aerobic oxidation of DPNH was proportional to the amount of mitochondria used. With a concentration of 0.1 mM DPNH and with 40–70  $\mu$ g of mitochondrial nitrogen as generally used, the rate of oxidation of DPNH was linear for at least 10 min.

Since the addition of 0.1 mM potassium cyanide completely inhibited aerobic oxidation of DPNH it is probable that cytochrome *c* oxidase acts as the terminal oxidase for this reaction. As shown in Table 1, the rate was stimulated by addition of heart-muscle cytochrome *c*, and this increased the apparent effect of inhibitors.

TABLE 1

EFFECT OF VARIOUS INHIBITORS ON THE DPNH OXIDASE ACTIVITY OF BEETROOT MITOCHONDRIA WITH AND WITHOUT ADDED CYTOCHROME *c*

Reaction mixtures, at pH 7.2, were equilibrated with air and contained 0.27M sucrose, 0.13 mM DPNH, 31 mM TRIS, 28 mM acetic acid, and 59  $\mu$ g of mitochondrial nitrogen per reaction mixture (3 ml). In experiment 2, heart-muscle cytochrome *c* (2  $\mu$ M) was added but this was omitted in experiment 1

Expt. No.	Control Rate ( $\Delta$ O.D./hr/mg N at 340 m $\mu$ )	Percentage Inhibition with:					
		Antimycin A (44 $\mu$ g)	Phenyl- urethane (2.7 mM)	Ethanol (2.7%, v/v)	Amytal (3.6 mM)	HOQNO (25 $\mu$ g)	SN 5949 (25 $\mu$ g)
1	27	57	43	—	46	—	—
2	95	80	69	29	77	65	62

Of the compounds investigated, antimycin A was the most inhibitory. Ethanol, in which both the antimycin A and the phenylurethane were dissolved, caused much less inhibition than either (Table 1).

*(b) Cytochrome c Oxidase Activity*

The aerobic oxidation of reduced heart-muscle cytochrome *c* (60  $\mu$ M) was completely inhibited by 0.1 mM potassium cyanide. There was a slight but variable inhibition with ethanol (2.7 per cent. v/v). There was no inhibition by antimycin A, phenylurethane, amytal, HOQNO, or SN 5949 when used at similar concentrations to those shown in Table 1, at which there is considerable inhibition of DPNH oxidase activity.

*(c) Cytochrome c Reductase Activity*

Table 2 shows the effect of a number of inhibitors on the rate of enzymic reduction of heart-muscle cytochrome *c* in the presence of 0.1 mM potassium cyanide. A slow rate of reduction of cytochrome *c* which occurred in the absence of substrate was insensitive to inhibitors.

With L-malate, the activity was markedly stimulated by addition of DPN, but there was no effect on activity with succinate.

Concentrations of inhibitors greater than those shown in Table 2 caused no further inhibition of the rates with DPNH or with succinate as substrates. Sodium malonate, in equimolar concentration with succinate (0.05M), caused almost complete inhibition of succinate-cytochrome *c* reductase activity.

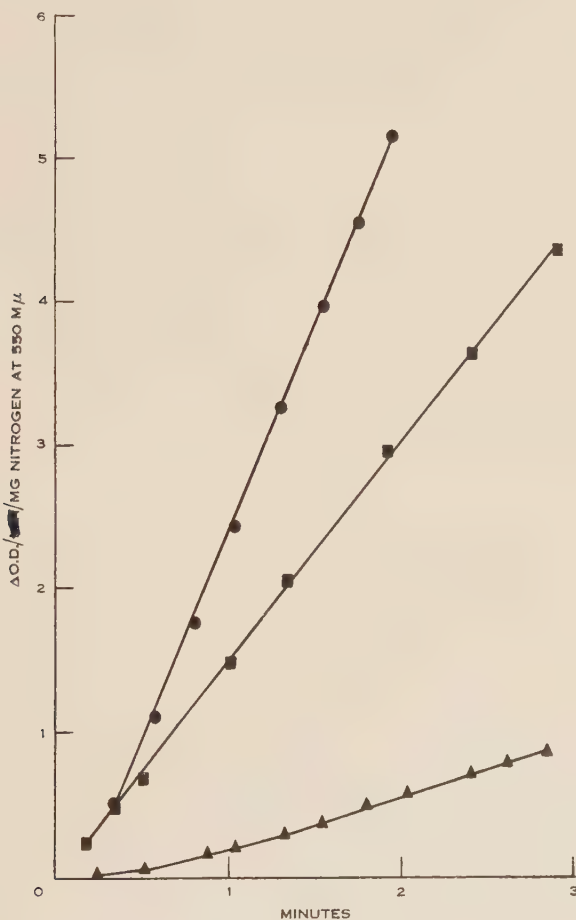


Fig. 1.—Effect of freezing and of hypotonic treatment on the succinate-cytochrome *c* reductase activity of beetroot mitochondria. Activities were determined at pH 7.2 and at 25°C under the conditions given in Table 2. ▲ Freshly isolated mitochondria; ■ after storage in 0.4M sucrose at -15°C for 3 days; ● after treatment with water for 30 min.

With succinate as substrate, there was consistently an initial lag of 1–2 min before a linear rate of reduction of cytochrome *c* was obtained. As shown in Figure 1, this lag was eliminated when the mitochondria were damaged structurally by freezing and thawing or by exposure to hypotonic solutions. These treatments also caused a marked stimulation of the activity.

*(d) Dichlorophenolindophenol (Dye) Reductase Activity*

Dye reduction was measured anaerobically with 0.1 mM potassium cyanide (Table 3). With succinate, the activity was low but was stimulated by addition of 5 mM EDTA (control, 31; with EDTA, 43 O.D. units/hr/mg nitrogen), possibly due to

TABLE 2  
EFFECT OF SOME INHIBITORS ON THE CYTOCHROME *c* REDUCTASE ACTIVITY OF BEETROOT MITOCHONDRIA WITH VARIOUS SUBSTRATES

Reaction mixtures, at pH 7.2, contained 0.27M sucrose, 31 mM TRIS, 28 mM acetic acid, 0.1 mM KCN, 60  $\mu$ M heart-muscle cytochrome *c*, and 59  $\mu$ g mitochondrial nitrogen per reaction mixture (3 ml), and with 0.26 mM DPNH or 50 mM sodium succinate or malate as substrates

Substrate	DPN Concn. (mM)	Control Rate ( $\Delta$ O.D./hr/mg N at 550 m $\mu$ )	Percentage Inhibition with:			
			Antimycin A (44 $\mu$ g)	Phenyl- urethane (2.7 mM)	Ethanol (2.7%, v/v)	Amytal (3.6 mM)
DPNH	0	192	49	28	16	43
Succinate	0	106	88	55	0	0
Malate	0	31	80	65	—	74
Malate	0.07	76	70	57	—	49
Malate	0.13	86	55	52	—	44

chelation of toxic material in the dye preparation. There was no effect of EDTA on the activity with DPNH. A non-enzymic reduction of dye by DPNH at about 5 per cent. of the enzymic rate was observed.

TABLE 3  
EFFECT OF INHIBITORS ON THE ENZYMIC REDUCTION OF 2,6-DICHLORO-PHENOLINDOPHENOL BY BEETROOT MITOCHONDRIA

Reaction mixture, at pH 7.2, contained 0.27M sucrose, 31 mM TRIS, 28 mM acetic acid, 0.1 mM KCN, 5 mM EDTA, 0.04 per cent. 2,6-dichlorophenolindophenol, either 0.26 mM DPNH or 50 mM sodium succinate, and 53  $\mu$ g of mitochondrial nitrogen per reaction mixture (3 ml)

Substrate	Control Rate ( $\Delta$ O.D./hr/mg N at 600 m $\mu$ )	Percentage Inhibition with:	
		Amytal (3.6 mM)	Malonate (0.03M)
DPNH	74	25	0
Succinate	43	0	69

At similar concentrations to those shown in Table 2, there was no inhibition by antimycin A, phenylurethane, or ethanol with either DPNH or succinate as

substrates. However, as shown in Table 3, amytal inhibited activity with DPNH, and malonate inhibited activity with succinate as substrate.

(e) *Effects of Storage and other Treatments on Enzymic Activities*

There was no decline in oxidase or reductase activities when freshly isolated suspensions of the mitochondria were kept in an ice-bath for 1-1½ hr. Freezing and thawing of suspensions stimulated all enzymic activities investigated. The increased activities were subsequently maintained by storage of the mitochondria in 0.4M sucrose at -15°C for several days, but declined after 17 days (Table 4).

TABLE 4  
EFFECT OF STORAGE AT -15°C ON THE ENZYMIC PROPERTIES OF BEETROOT  
MITOCHONDRIA IN 0.4M SUCROSE

Reaction mixture, at pH 7.2, contained 0.27M sucrose, 31 mM TRIS, 28 mM acetic acid, 0.26 mM DPNH or 50 mM sodium succinate where indicated, and 43 µg of mitochondrial nitrogen per reaction mixture (3 ml). The following additions were made for the enzymic activities indicated: DPNH oxidase, 2 µM heart-muscle ferricytochrome *c*; cytochrome *c* reductase, 60 µM heart-muscle ferricytochrome *c* and 0.1 mM KCN; cytochrome *c* oxidase, 60 µM ferrocytochrome *c*; dichlorophenolindophenol (dye) reductase, 0.04 per cent. 2,6-dichlorophenolindophenol, 5 mM EDTA, and 0.1 mM KCN

Enzymic System	Activity (Δ O.D./hr/mg N)		
	On Isolation	3 Days Storage	17 Days Storage
DPNH oxidase*	53	101	87
DPNH-cytochrome <i>c</i> reductase†	192	275	241
Succinate-cytochrome <i>c</i> reductase‡	104	(85)§	170
Cytochrome <i>c</i> oxidase†	122	133	90
DPNH-dye reductase‡	154	328	114
Succinate-dye reductase‡	34	65	58

\* Measured at 340 mµ.

† Measured at 550 mµ.

‡ Measured at 600 mµ.

§ This result is atypical; usually an increase of activity as compared with the control was obtained, as in Table 5.

A suspension of fresh mitochondria in 0.4M sucrose was centrifuged and the sedimented mitochondria were resuspended in water for 5 min at 0°C and then sucrose was added and the suspension made up to the original volume in 0.4M sucrose. As shown in Table 5, this hypotonic treatment caused a marked stimulation of the enzymic activities, comparable with that obtained by freezing and thawing.

(f) *Oxidative Phosphorylation*

As shown by Table 6, the mitochondria catalyse esterification of inorganic phosphate coupled to oxidation of succinate or malate. Addition of DPN stimulated



oxygen uptake with malate but depressed the P/O ratio. Storage of the mitochondria in 0.4M sucrose at  $-15^{\circ}\text{C}$  for 7 days caused a decline in oxygen uptake and in the P/O ratios with malate alone, and with malate and DPN.

TABLE 5  
COMPARISON OF FREEZING AND HYPOTONIC TREATMENTS ON THE ENZYMIC ACTIVITIES OF BEETROOT MITOCHONDRIA

Reaction mixture, at pH 7.2, contained 0.27M sucrose, 31 mM TRIS, 28 mM acetic acid, 0.26 mM DPNH or 50 mM sodium succinate where indicated, and 43  $\mu\text{g}$  of mitochondrial nitrogen per reaction mixture (3 ml). The following additions were made for the enzymic activities indicated: DPNH oxidase, 2  $\mu\text{M}$  heart-muscle ferricytochrome *c*; cytochrome *c* reductase, 60  $\mu\text{M}$  heart-muscle ferricytochrome *c* and 0.1 mM KCN; cytochrome *c* oxidase, 60  $\mu\text{M}$  ferrocytochrome *c*; dichlorophenolindophenol (dye) reductase, 0.04 per cent. 2,6-dichlorophenolindophenol, 5 mM EDTA, and 0.1 mM KCN

Enzymic System	Activity ( $\Delta$ O.D./hr/mg N)		
	Control	After Freezing Treatment	After Hypotonic Treatment
DPNH oxidase	52	110	119
DPNH-cytochrome <i>c</i> reductase	150	259	290
Succinate-cytochrome <i>c</i> reductase	36	150	174
Cytochrome <i>c</i> oxidase	108	330	570
DPNH-dye reductase	154	224	254

TABLE 6  
ESTERIFICATION OF INORGANIC PHOSPHATE COUPLED WITH OXIDATION OF SUCCINATE AND MALATE BY BEETROOT MITOCHONDRIA

Reaction mixture (3 ml) contained 0.27M sucrose, 31 mM TRIS, 28 mM acetic acid, 2  $\mu\text{M}$  heart-muscle cytochrome *c*, 13 mM  $\text{K}_2\text{HPO}_4$ , 1 mM ADP, 6.7 mM  $\text{MgCl}_2$ , 10 mM NaF, 20 mM glucose, 50 mM sodium succinate or malate, and excess hexokinase. Oxygen uptake was determined manometrically at  $25^{\circ}\text{C}$

Substrate	Oxygen Uptake ( $\mu\text{g}$ -atoms)	ATP Formed† ( $\mu\text{moles}$ )	P/O Ratio
Succinate	6.7	5.2	0.78
Malate	6.7	13.4	2.00
Malate + 0.48M DPN	9.9	13.9	1.40
Malate*	2.2	3.7	1.68
Malate + 0.48 mM DPN*	5.1	6.1	1.20

\* Mitochondrial preparation stored for 7 days at  $-15^{\circ}\text{C}$ .

† Equivalent to  $\mu\text{g}$ -atoms of inorganic phosphate esterified; ATP estimated as  $\mu\text{moles}$  of glucose 6-phosphate formed.

Figure 2 shows that with freshly isolated mitochondria, the rate of oxygen uptake with succinate alone was linear for about 30 min but then declined. How-

ever, under the conditions necessary for oxidative phosphorylation, the oxygen uptake was linear for at least 80 min.

### (g) Cytochrome Pigments

Direct spectroscopic observations of pellets of sedimented mitochondria and suspensions in 0.4M sucrose were made with a low-dispersion spectroscope. Materials were observed at about 0°C and after cooling to about -190°C in liquid air. With pellets and heavy suspensions of particles it has been found unnecessary to use 50 per cent. glycerol for intensification of the absorption bands at -190°C (cf. Keilin and Hartree 1949).

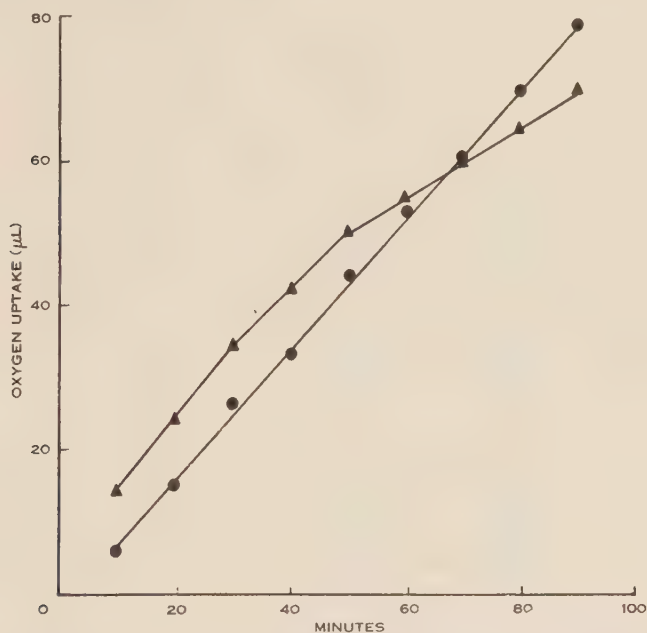


Fig. 2.—Oxidation of succinate by beetroot mitochondria. Activities were determined manometrically (at 25°C) with air as the gas phase. The reaction mixture (3 ml, pH 7.2) contained 0.27M sucrose, 31 mM TRIS, 28 mM acetic acid, 2  $\mu$ M heart-muscle cytochrome *c*, and 50 mM sodium succinate and 370  $\mu$ g of mitochondrial nitrogen. ▲ Control (no additions); ● with 1 mM ADP, 6.7 mM  $MgCl_2$ , 10 mM NaF, 20 mM glucose, 13 mM potassium phosphate, and excess hexokinase.

When freshly isolated, beetroot mitochondria are chocolate-brown in colour, and show no distinct absorption bands of haemoprotein pigments which are probably oxidized. On occasions, a weak broad band between 535 and 550  $m\mu$  is seen. The mitochondria also show a strong, broad band near 490  $m\mu$  which, however, does not change with oxidation and reduction and is probably not due to a haemoprotein. This component occurs in mitochondria from silver-beet petioles and from wheat roots (Martin and Morton 1957).

When reduced with succinate anaerobically at 0°C, the mitochondria were pale brown and showed asymmetric absorption areas between 518 and 530  $m\mu$ , between

550 and 562  $m\mu$ , and at about 595–598  $m\mu$ . These bands were much intensified by addition of sodium dithionite. The broad band between 550 and 562  $m\mu$  was resolved into three bands, attributed to the  $\alpha$ -absorption bands of cytochrome *b* (558–562  $m\mu$ ), cytochrome *c*<sub>1</sub> (552–555  $m\mu$ ), and cytochrome *c* (550  $m\mu$ ). The resolution of these bands is assisted by examination after treatment with liquid air ( $-190^{\circ}\text{C}$ ). However, this is also accompanied by a slight shift of the band positions towards lower wavelengths as well as a “splitting” of certain bands, so that identification of components is more complicated than by examination at  $0^{\circ}\text{C}$ .

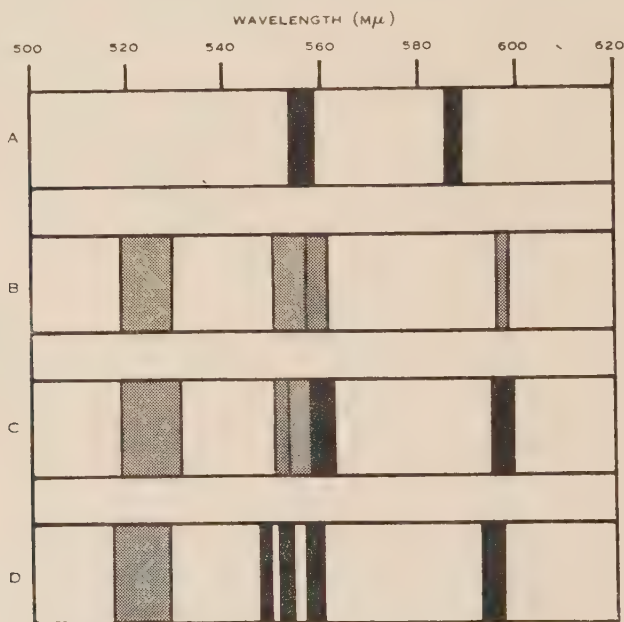


Fig. 3.—Diagrammatic representation of visible absorption bands of beet mitochondrial haem pigments: *A*, with alkaline pyridine and sodium dithionite at  $0^{\circ}\text{C}$ ; *B*, with sodium succinate and anaerobic conditions at  $0^{\circ}\text{C}$ ; *C*, with sodium dithionite at  $0^{\circ}\text{C}$ ; *D*, with sodium dithionite at  $-190^{\circ}\text{C}$ .

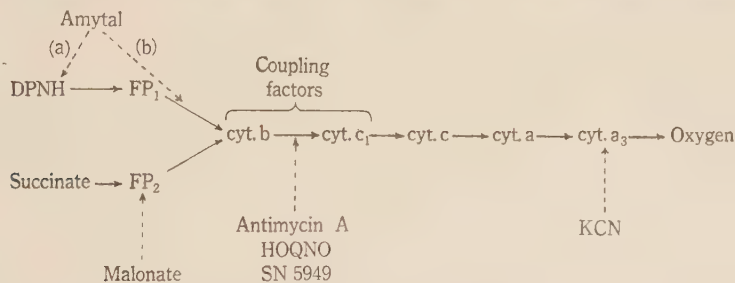
After treatment of the mitochondria with alkaline pyridine and sodium dithionite, intense  $\alpha$ - and  $\beta$ -absorption bands of the pyridine haemochromes of protohaem (at 557  $m\mu$ ) and of haem *a* (at 588  $m\mu$ ) were observed, as well as a very weak  $\alpha$ -band of the haemochromogen of haem *c* (near 550  $m\mu$ ). Figure 3 shows the relative positions and relative intensities of the absorption bands observed.

#### IV. DISCUSSION

The beetroot mitochondria used in this work appeared to be substantially free of contamination by plastids and microsomes, when electron micrographs of preparations fixed with osmium tetroxide were examined. The observations therefore relate only to mitochondria.

The spectroscopically detectable haemochromes have been identified as cytochromes ( $a+a_3$ ),  $b$ ,  $c_1$ , and  $c$  (Fig. 3), similar to the cytochromes of mitochondria from beet petioles and wheat roots (Martin and Morton 1957). However, these components are not necessarily identical with the analogous cytochromes of mitochondria from animal tissues. Lundegårdh (1952, 1958) and Martin and Morton (1957) identified cytochrome  $a_3$  in plant tissues and this finding is supported by numerous reports of the complete inhibition of respiratory activity by potassium cyanide (0.1 mM) as found in this work. The absorption band at 595–598  $m\mu$  (Fig. 3) is therefore attributed to cytochrome ( $a+a_3$ ) and this is regarded as the terminal component of the respiratory chain. As compared with wheat-root mitochondria, beetroot mitochondria have a relatively large amount of cytochrome  $a$ -type component and relatively small amount of cytochrome  $c$ . This is consistent with the observed stimulation of DPNH oxidase activity by added cytochrome  $c$  (Table 1).

The component with an  $\alpha$ -band at about 554  $m\mu$  (Fig. 3) is similar to that observed in other plant mitochondria and called by Martin and Morton (1957) "cytochrome  $c_1$ ". Like cytochrome  $b$  ( $\alpha$ -band 558–562  $m\mu$ ) it is believed to be a component of the respiratory chain, since it is reduced by DPNH and succinate. A similar component has been observed by other workers (Crane 1957; Hackett and Haas 1958; and Lundegårdh 1958). Until this cytochrome is isolated, and the nature of the prosthetic group is identified, the name "cytochrome  $c_1$ " must be regarded as tentative and not as implying identity with cytochrome  $c_1$  of animal tissues (see Morton 1958). From these observations, the respiratory chain\* of the beetroot mitochondria is assumed to comprise the following components at least:



Here, FP<sub>1</sub> and FP<sub>2</sub> represent different flavoproteins.

The probable sites of action of inhibitors are indicated. Whereas Ernster *et al.* (1955) consider amytal to act between the flavoprotein (FP<sub>1</sub>) and cytochrome  $b$  (site (b)), Chance and Williams (1956) consider it to act between DPNH and flavoprotein (site (a)). The site of action of antimycin A was identified by Potter and Reif (1952) for animal tissue and by Martin and Morton (1957) for plant mitochondria. HOQNO (Lightbown and Jackson 1956; Jackson and Lightbown 1958) and SN 5949 (Ball, Anfinsen, and Cooper 1947) each appear to act at a site similar to that at which antimycin A acts in animal tissues. Chance and Hackett (1959) have recently described the effects of HOQNO and some other inhibitors on the

\* Lester and Crane (1959) have reported substantial amounts of naphthoquinones related to ubiquinone (R. A. Morton 1958) in plant mitochondria; whether ubiquinone is a component of the respiratory chain is unknown.



respiratory chain of mitochondria of skunk cabbage (*Symplocarpus foetidus*). Chance and Sacktor (1958) and Chance (1958) have observed a substantial increase in the apparent amount of cytochrome *b* of animal mitochondria when inhibited with antimycin A, or reduced with dithionite, as compared with substrate reduction anaerobically. This behaviour is particularly apparent with plant mitochondria (Martin and Morton, unpublished data). Antimycin A may combine with cytochrome *b* itself.

The activities of individual enzymic steps of the respiratory chain have been compared by assuming that the oxidation of DPNH involves two hydrogen equivalents and the reduction of cytochrome *c* only one, and by expressing all rates as  $\mu$ moles of oxygen uptake/hr/mg mitochondrial nitrogen, as shown in Table 7.

TABLE 7  
COMPARISON OF THE OXIDATION CAPACITY OF VARIOUS ENZYME SYSTEMS OF  
BEETROOT MITOCHONDRIA

Assay details for each of the systems are given in the text and in Tables 1-6. The mean and range of values (shown in parenthesis) are for determinations on at least five different preparations

System	Wavelength for Estimation ( $m\mu$ )	$\Delta$ O.D./hr/mg N	Oxygen Consumed ( $\mu$ l/hr/mg N)
DPNH oxidase	340	63	340 (286-378)
DPNH-cytochrome <i>c</i> reductase	550	155	133 (105-165)
Succinate-cytochrome <i>c</i> reductase	550	87	75 ( 48- 96)
Succinate oxidase	—	—	472 (314-637)
Cytochrome <i>c</i> oxidase	550	116	99 ( 84-116)
DPNH-dye reductase	600	157	276 (188-342)
Succinate dehydrogenase	600	37	65 ( 56-144)
Malate oxidase	—	—	233 (206-301)
Malate oxidase*	—	—	323 (257-445)

\* With 0.48 mm DPN (see Table 6).

Under the conditions of the experiments, the rate of oxidation of all substrates is a maximum when a small concentration of cytochrome *c* is added and oxygen is the terminal acceptor. Moreover, the maximum inhibition of DPNH oxidation by antimycin A, phenylurethane, and amytal occurs under these conditions (Table 1). High concentrations of added cytochrome *c* interfere with the normal sequence of reactions as indicated by the decreased inhibition of DPNH-cytochrome *c* reductase (Table 2) when compared with DPNH oxidase activity (Table 1), as well as by the lower rate of substrate oxidation with cytochrome *c* as the terminal acceptor (Table 7). The lower inhibition of the cytochrome *c* reductase activities (Table 2) is not due to binding of the inhibitors by added cytochrome *c*, since Reif and Potter (1953) have shown that cytochrome *c* does not bind antimycin A. Moreover, increasing the concentrations of inhibitors did not increase the percentage inhibition, and succinate-cytochrome *c* reductase activity was 88 per cent. inhibited by the antimycin A (Table 2).

It is possible that the mitochondria had lost some cytochrome *c* by extraction during isolation. This would account for the very small amount of cytochrome *c* detected spectroscopically, and for the marked influence of added cytochrome *c* on the enzymic activities. Mitochondria prepared by Martin and Morton (1957) from silver-beet petioles and from wheat roots also had relatively small amounts of cytochrome *c*.

In beetroot mitochondria, as in mitochondria from other plants (Martin and Morton 1956*a*, 1956*b*), there appear to be at least two pathways for oxidation of DPNH, one of which is sensitive, the other insensitive, to antimycin A. The pathway insensitive to antimycin A is stimulated by addition of large amounts of heart-muscle cytochrome *c* or of DPNH, and it is possible that it represents an "external" or non-phosphorylating system for oxidation of DPNH in solution as distinct from "bound" DPNH of mitochondria (cf. Lehninger 1951, Ernster *et al.* 1955).

The inhibitory effect of ethanol observed in these experiments was not specific for any one step in the electron transport chain. Beer and Quastel (1958) reported that dilute ethanol partially inhibited the salt-stimulated respiration of brain cortex slices but had no effect on the respiration of the isolated mitochondria. Keilin and Hartree (1940) found that a high concentration of ethanol (45 per cent.) irreversibly modified the cytochromes *a*<sub>3</sub>, *a*, and *b* and thus completely inhibited their succinate-cytochrome *c* reductase system. A similar effect could have occurred to the beet mitochondrial system studied here, but to such a small degree that it was noticeable only with the efficient DPNH oxidation process.

The stimulation of all enzymic systems of beetroot mitochondria by freezing and thawing and by exposure to hypotonic conditions (Table 4 and 5) is probably due to the improved permeability to substrates. Whereas with freshly isolated mitochondria there is a pronounced lag in the reduction of cytochrome *c* by succinate, this is not observed in the modified mitochondria (Fig. 1). The stability of the enzymic systems of beetroot mitochondria including those systems involved in oxidative phosphorylation is particularly noteworthy. Whereas Martin and Morton (1956*b*) observed that the succinate dehydrogenase of mitochondria from wheat root rapidly lost activity on storage, the beetroot mitochondria retain activity when held at -15°C for some days. These differences probably reflect small differences in the structure of the mitochondria from the two sources and emphasize the caution necessary in extending results obtained with one plant tissue to another tissue of the same plant, or to a similar tissue of a different species.

#### V. ACKNOWLEDGMENTS

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# INFLORESCENCE INITIATION IN *LOLIUM TEMULENTUM* L.

## I. EFFECT OF PLANT AGE AND LEAF AREA ON SENSITIVITY TO PHOTOPERIODIC INDUCTION

By L. T. EVANS\*

[Manuscript received October 5, 1959]

### Summary

As they increase in age, plants of *L. temulentum* require exposure to progressively fewer long days before they can initiate inflorescences, until, after being grown for 5 weeks at 25°C for 8 hr and at 20°C for the remainder of each day, they require only one long day. This increase in sensitivity to photoperiodic induction with age varies to a slight degree with seasonal light conditions. In young plants, exposure to long days for less than the period required for inflorescence initiation increases the rates of leaf initiation and apex elongation.

When the leaf areas of mature plants are reduced by removal of all the lower leaves, exposure of only a few square centimetres of leaf blade to one day of continuous light is sufficient for inflorescence initiation. On the other hand, when only the lower leaves are left the plants require exposure to the same number of long days as do younger plants with the same leaves present.

It is concluded that the expansion of leaves of higher ontogenetic rank contributes more to the increase in sensitivity to photoperiodic induction than do increase in total leaf area, leaf age, or apex age of the plants.

### I. INTRODUCTION

Mature plants of *Lolium temulentum* L. have been shown (Evans 1958) to require exposure to only one long day for the initiation of inflorescence differentiation. It has also been found that only a few square centimetres of one leaf need be exposed to this long day, and that the intensity of light during the supplementary period of illumination need be only 1-2 f.c. for inflorescence initiation to occur. This pre-eminent sensitivity of the species to induction by long days, which renders it quite comparable to *Xanthium pensylvanicum* among short-day plants, is not present in young plants, however, and is only attained after about 5 weeks of growth.

Seedlings and young plants of many short-day species are known to require more short days for photoperiodic induction than are mature plants (Borthwick and Parker 1938, 1940; Moshkov 1939; Harder and von Witsch 1942; Lona 1949). Among long-day plants, Chouard (1950) has shown that *Scabiosa ukrainica* needs exposure to 3 weeks of continuous light at 3 months of age, but to only 1 week at 9 months of age. Juvenile insensitivity to photoperiodic induction thus appears to be a fairly widespread phenomenon, although there are exceptions such as *Arachis hypogaea* (Rossem and Bolhuis 1954). It is the purpose of the present paper to analyse the factors responsible for the increase in sensitivity to photoperiodic induction with age in *L. temulentum*.

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## II. METHODS

All plants were grown singly in small plastic pots containing either vermiculite or perlite, and were given Hoagland's nutrient solution and water daily. The pots were held in boxes providing automatic control of the photoperiod to a length of 8 hr. The boxes were kept in a glass-house whose temperature was maintained at approximately 25°C for 8 hr and at 20°C for the remainder of each day (designated 25°C/20°C). Departures from the set temperature conditions were not great, and the main variation between experiments was probably in seasonal light intensity and spectral composition. Leaves on the main stem of all plants were notched for identification as they appeared. At various times groups of plants were removed and exposed to one or more days of continuous light, each consisting of daylight

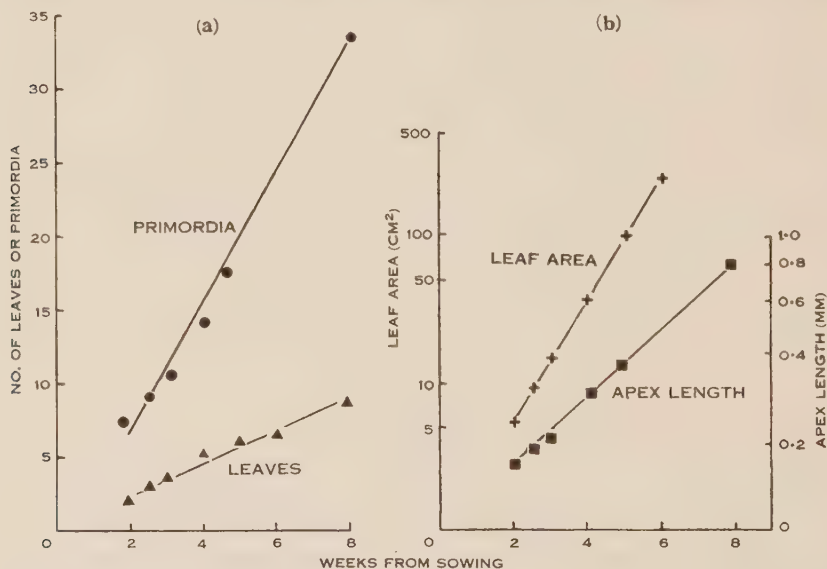


Fig. 1.—Course of growth in *L. temulentum* plants at 25°C/20°C in 8-hr photoperiods of summer daylight.

for 8 hr (at 25°C) followed by illumination of 10–15 f.c. intensity at plant height from incandescent sources (at 20°C). They were then returned to the short-day boxes for 2 or 3 weeks before dissection. At dissection the state of differentiation of the main shoot apex and its length were recorded, together with the number of leaves and of nodes to flowering, and the total number of primordia present.

## III. RESULTS

(a) *Change in Photoperiodic Sensitivity with Chronological Age*

In all, five age-sensitivity series have been examined. These were grown at different seasons, and the results of the most complete series will be described first. This series was sown in January, with eight plants in each treatment group. The course of growth in 8-hr photoperiods at 25°C/20°C may be seen from Figure 1, from

which it is evident that leaf area increased exponentially throughout the experimental period, while leaf appearance and leaf initiation increased approximately linearly at rates of one per 6.4 and 1.7 days respectively. Thus, about three-quarters of the initiated leaf primordia accumulated at the shoot apex under these conditions, with consequent increase in its length.

TABLE 1

EFFECT OF PLANT AGE AND OF NUMBER OF LONG DAYS ON THE PERCENTAGE OF *L. TEMULENTUM* PLANTS WHICH INITIATED INFLORESCENCES, AND ON THE NUMBER OF NODES TO FIRST FLOWERING  
Plants dissected 2 weeks after exposure to the first long day

	Age of Plant (days)	Days of Continuous Light:						
		0	1	2	3	4	6	8
Plants (%)	14	0	0	0	0	75	100	100
Nodes						7.5	7.0	6.6
Plants (%)	18	0	0	0	25	100	100	100
Nodes						7.8	7.4	7.0
Plants (%)	21	0	0	62.5	87.5	100	—	100
Nodes				9.3	8.8	8.0		8.0
Plants (%)	28	0	0	100	100	100		
Nodes				9.3	9.0	8.9		
Plants (%)	34	0	100	100	100			
Nodes			10.6	10.3	9.5			
Plants (%)	42	0	100	100				
Nodes			11.7	11.1				

The results of the dissections made 2 weeks after exposure to the first long day are given in Table 1 and in Figures 2 and 3. Plants which were only 2 weeks old apparently required exposure to more than 4 days of continuous light before they all initiated inflorescences, while plants 18, 21, 28, and 34 days old at their first exposure to long days required only 4, 4, 2, and 1 days respectively (Table 1).

The results in Table 1 also indicate that the number of nodes to flowering tends to be lower the more inductive long days given, and the younger the plants at induction. The lowest values for each age are very similar to those obtained by Cooper (1956) with plants of *L. temulentum* of equivalent leaf-appearance age. Comparison of these numbers with the numbers of primordia that were present at the time of inflorescence induction (Fig. 1) indicates that, in all except the youngest plants, a proportion of the initiated leaf primordia must ultimately subtend spikelet primordia, this proportion increasing markedly with advance in age of the plants and with the number of long days given.

The data given in Figure 2 indicate that apex length increased with the number of long days given in all cases, even when no long day effect was manifest in the proportion of plants which had initiated inflorescences. Similarly it is evident from Figure 3 that the rate of initiation of primordia increased with increase in exposure to long days even when the number of long days given was insufficient to elicit the differentiation of double ridges.

(b) *Variations in Sensitivity not due to Chronological Age*

The results of the other age-sensitivity series were all similar in pattern, but in only one case were they identical to the series already described. In the others, particularly in those grown during the winter months, plants were less sensitive to induction than plants of the same chronological age in the series described. They were also smaller in leaf area, and had initiated fewer primordia, the plastochrone

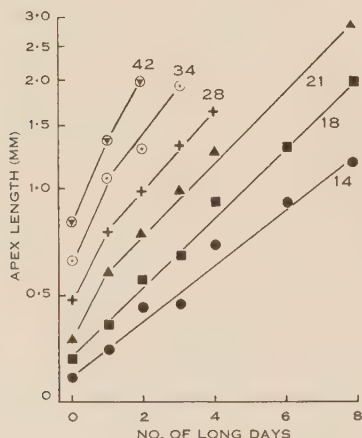


Fig. 2

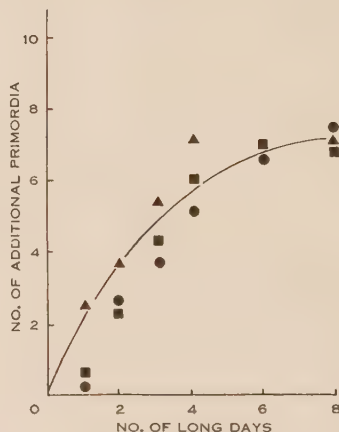


Fig. 3

Fig. 2.—Effect of plant age on apex length 2 weeks after exposure to the first of a number of days of continuous light. Age of plants in days indicated on the figure.

Fig. 3.—Number of primordia differentiated in excess of the number in plants not exposed to long days, 2 weeks after exposure to the first long days. Age of plants: ● 14 days; ■ 18 days; ▲ 21 days.

being up to 3 days, about twice the interval in the series described. The rate of leaf appearance was less affected, the slowest rate being one every 9.6 days, compared with 6.4 days in the series described.

Since all series were grown under the same conditions of temperature, photoperiod, and nutrient culture, the differences between them are probably due to seasonal changes in the intensity or quality of daylight.

Bases other than chronological age were therefore used to compare the results of the various series. Total leaf area, number of primordia initiated, and number of expanded leaves were used, but only with the last were the results at all

comparable. In fact, the change in sensitivity to photoperiodic induction with increase in the number of expanded leaves on the primary shoot was almost identical in all series. This suggests that it is the expansion of the later-formed leaves which most affects the sensitivity of *L. temulentum* plants to induction by long days, a conclusion which is supported by the results given below.

(c) *Minimum Leaf Area Required for Induction by One Day of Continuous Light*

The plants were sown in early April, and grown for 41 days until leaf 6 on the primary shoot was fully expanded. All other leaf blades were then removed from the plants, and the blade of leaf 6 was reduced to varying degrees, in groups of 8–10 plants, just before exposure to a single day of continuous light. The plants were dissected after a further 3 weeks in the standard short-day conditions, with the results shown in Figure 4. Exposure of only 6 cm<sup>2</sup> of leaf 6 to one long day was

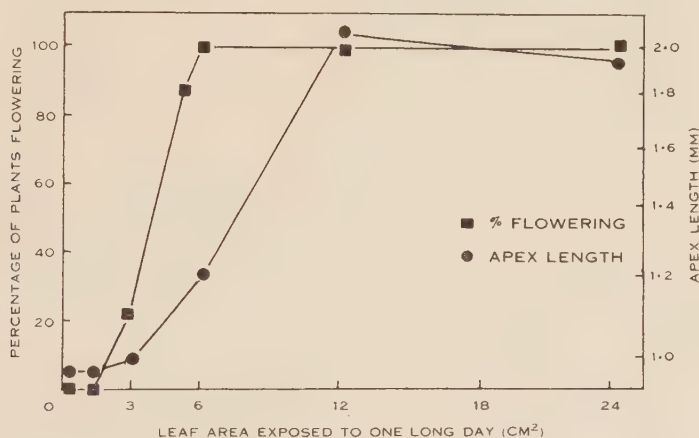


Fig. 4.—Effect of the area of leaf 6 exposed to one day of continuous light on the percentage of plants initiating inflorescences (■) and on increase in length of the shoot apex (●).

apparently sufficient for the initiation of inflorescences in all plants, and exposure of 12 cm<sup>2</sup> resulted in the maximal rate of inflorescence development under these conditions. Since the minimum effective leaf area is comparable to the leaf area of plants 14 days old, the increase in photoperiodic sensitivity with age cannot be ascribed to increasing leaf area *per se*.

(d) *Effect of Reduced Leaf Areas on the Photoperiodic Sensitivity of Mature Plants*

A large number of plants was sown late in April to 8-hr photoperiods at 25°C/20°C and subsequently divided at random into three groups. Group A comprised an age-sensitivity series, like those already described, for comparison with the other two groups. Lots of eight plants were removed at intervals from the short-day conditions, exposed to a number of days of continuous light, and returned to short days until dissection 2 weeks after the first exposure to long days.



The group B plants were kept under short-day conditions until 35 days old, by which time leaf 6 was fully grown. Their leaf areas were then reduced to areas equivalent to those of the younger plants in group A at the time of their exposure to long days, and the plants were then given the same long-day treatments as the group A plants, in lots of 8. In the group B plants the reductions in leaf area were made by removal of the younger leaves, with the result that the same leaves were left on the main shoots as were present on the younger plants at the equivalent age.

TABLE 2

PERCENTAGE OF PLANTS WITH INITIATED INFLORESCENCES 14 DAYS AFTER THE INITIAL EXPOSURE TO LONG DAYS

Age of Plant (days)	Leaf Area Exposed to Long Days (cm <sup>2</sup> )	Leaves or Parts of Leaves Left	Number of Long Days at 25°C/20°C				
			0	1	2	3	4
14	5.0	All (leaf 1, leaf 2)	0	0	0	0	75
35	5.0	Lower (leaf 1, leaf 2)	0	0	0	37.5	100
35	5.0	Upper (leaf 6)	0	87.5	100	—	—
18	8.6	All (leaves 1-3)	0	0	0	37.5	100
35	8.6	Lower (leaves 1-3)	0	0	12.5	37.5	87.5
35	8.6	Upper (leaf 6)	0	100	100	—	—
21	13.2	All (leaves 1-3)	0	0	62.5	100	—
35	13.2	Lower (leaves 1-3)	0	0	37.5	100	—
35	13.2	Upper (leaf 6)	0	100	100	—	—
28	36.3	All (leaves 1-5)	0	0	100	100	—
35	36.3	Lower (leaves 1-5)	0	0	100	—	—
35	36.3	Upper (leaf 5, leaf 6)	0	100	100	—	—
35	95.5	All (leaves 1-6)	0	100	100	100	—

In the group C plants, on the other hand, all the lower leaf blades were removed, and the required leaf areas were derived from the two uppermost fully expanded leaves of the main stem. Otherwise, the treatment of the plants was the same as that for group B.

Additional leaf areas developing during the long-day treatments were removed daily. The primary shoots of the plants were dissected 14 days after the beginning of the long-day treatment, and some of the results of these dissections are given in Table 2.

The increase in sensitivity to long-day induction with advance in age of the group A plants is closely similar to that already described. So too, is that for the mature plants in which only the lower leaves were exposed to long days (group B), there being only minor differences between the two groups despite the increased age of both the leaves and the differentiating shoot apex in the latter. On the other hand, as would be expected from the results given in Section III(c), the equivalent

areas of the upper leaves were apparently far more effective in induction, since exposure to only one long day was sufficient for inflorescence initiation in virtually all of the group C plants.

#### IV. DISCUSSION

The progressive increase in sensitivity to photoperiodic induction with age of plants of *L. temulentum* could have several explanations, and the evidence bearing on some of these will now be considered. Lona (1949) and Zeevaart (1958) have previously discussed the results for short-day plants.

##### (a) *Increase in the Perceptive Leaf Area of the Whole Plant*

In *Oenothera biennis* (Chouard 1950) there is evidence that the total leaf area of the plants may limit sensitivity to photoperiodic induction. However, plants of *L. temulentum*, of an age just to have attained full sensitivity to photoperiodic induction, may retain that sensitivity even when their leaf area is reduced by 95 per cent. Hence, as in the several other species known to behave similarly in this respect, it cannot be the total leaf area which limits sensitivity to photoperiodic induction in young plants.

##### (b) *Increase in the Photoperiodic Sensitivity of Individual Leaves as they become Older*

From their experiments with *Xanthium*, Khudairi and Hamner (1954) and Salisbury (1955) suggest that although leaves may increase in their sensitivity to photoperiodic induction until they are almost fully expanded they tend thereafter to decline in sensitivity with increased age. However, since they took the position of a leaf as the criterion of its age, effects due to the age of a leaf and those due to its position are confounded.

From grafting experiments with *Perilla*, Zeevaart (1958) found that fully expanded leaves retain their photoperiodic sensitivity for a considerable time, and that the age of leaves was far less important than their position on the plant. It seems likely that, in *L. temulentum* also, fully expanded leaves retain their photoperiodic sensitivity for some time, since the response to a number of long days of mature plants with only their basal leaves remaining was almost identical to that of young plants with the same, but much younger, leaves.

In no species is there any evidence of an increase in the photoperiodic sensitivity of fully expanded leaves with age.

##### (c) *Greater Photoperiodic Response in later-formed Leaves*

With a few exceptions (Zieriacks 1952; Kujirai and Imamura 1958), cotyledons appear to be unable to induce plants to flower under the appropriate photoperiodic conditions, and it is possible that the first-formed leaves may also be less sensitive than later ones to photoperiodic induction. In a series of elegant grafting experiments with *Perilla*, in which he could exclude effects due to the age of leaves or growing points, Zeevaart (1958) found that the lower leaves required exposure to more short days than did the later-formed ones before they could initiate flowering in vegetative stocks.

Since grafting experiments are precluded in *L. temulentum*, comparisons of the inductive efficiencies of various leaves are confounded with differences in their age. However, it was concluded above that leaf age did not appear to have a major effect on the sensitivity of the leaves to long-day induction, in which case the very great differences found between the sensitivity of the lower and that of the upper leaves is likely to be due to the position of the leaves on the plant, a conclusion which agrees with Zeevaart's results, and with the analysis of seasonal changes in the rate at which *L. temulentum* plants attain full sensitivity to long-day induction.

(d) *Increase in the Ability of the Growing Point of the Potential Inflorescence to Respond to the Floral Stimulus*

Crucial experiments to establish the contribution of increasing bud age to the increasing sensitivity to photoperiodic induction have not been done, and are precluded in the grasses because one would have to graft induced leaves of equal ontogenetic rank, age, and area to vegetative stocks of various ages. It is possible that plants with terminal inflorescences differ from those with axillary ones in this respect.

In soybeans, Borthwick and Parker (1940) found that neither the distance nor the direction of the induced leaf from the differentiating bud was of much account, which excludes effects due to minor differences in bud age and in distance over which the stimulus must be translocated.

In *L. temulentum*, the fact that the results obtained with mature plants on which had been left only the first-formed leaves agreed closely with those for the younger plants with the same leaves suggests that the shoot apex had not increased in responsiveness with age, unless the leaves had at the same time decreased in photoperiodic sensitivity to an equivalent degree.

It would seem, then, that it is the expansion of leaves of higher ontogenetic rank which contributes most to the increased sensitivity of older plants of *L. temulentum* to induction by long days, although an effect due to the increased age of the shoot apex is not altogether excluded.

## V. ACKNOWLEDGMENTS

I wish to acknowledge the technical assistance of Mrs. Katie Bretz in these experiments, and I am indebted to Drs. L. A. T. Ballard and R. F. Williams for discussion of the results.

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# ON THE UTILIZATION OF $\gamma$ -AMINO BUTYRIC ACID BY WHEAT SEEDLINGS

By A. H. G. C. RIJVEN\*

[Manuscript received November 27, 1959]

## Summary

Feeding experiments and enzyme tests were carried out to investigate the utilization of  $\gamma$ -aminobutyric acid (ABA) by wheat seedlings.

In the feeding experiments embryos were excised from the grain 20–24 hr after soaking, explanted on a basal medium to which different nitrogen sources were added, and incubated at 25°C.

ABA nitrogen was utilized for protein synthesis. The optimal concentration was 6.0 mM. Different amino acids tested at equivalent nitrogen concentration produced the following sequence in insoluble nitrogen content per seedling: control = L- $\alpha$ , $\gamma$ -diaminobutyric acid < DL- $\alpha$ -aminobutyric acid = ABA < L-proline < L-alanine = L-glutamic acid < L-asparagine = L-glutamine = nitrate.

Growth in length of the first leaf was on all occasions stimulated by ABA, but root growth was not.

Extracts of roots of 2- and 3-day-old intact seedlings had about 2.5 times the glutamic acid decarboxylase activity (per seedling) as that of shoot extracts; on a protein basis the ratio became more than 6.

No ABA-glutamic acid transaminase could be shown in root and shoot extracts, even after attempts to induce the enzyme, but alanine-glutamic acid transaminase activity was high in similar extracts. Neither was any evidence found for the presence of ABA oxidase in the seedlings.

## I. INTRODUCTION

$\gamma$ -Aminobutyric acid (ABA) has become recognized as a common component of the soluble nitrogen fraction of plants, but it is not found as a constituent of proteins.

ABA is of interest because of its relationship with the key metabolite, glutamic acid, from which it may be formed by decarboxylation. A puzzling aspect of the role of this amino acid is that the decarboxylases described do not favour carboxylation (Meister 1957), and the question has arisen whether this amino acid is a catabolic side product. In leaves of barley ABA content increases under anaerobic conditions (Naylor and Tolbert 1956). Kretovich and Yakovleva (1959) found a notable increase in ABA content after infiltrating potassium or ammonium  $\alpha$ -ketoglutarate into leaves of wheat seedlings, but they question the conversion of ABA to glutamic acid. In a number of lower plant forms ABA has been shown to be an active intermediate, but its role in higher plants is still not clear.

Re-utilization of ABA nitrogen can be envisaged by means of transamination. Miettinen and Virtanen (1953) showed that ABA may act as a donor substrate in a transamination reaction with  $\alpha$ -ketoglutarate in pea root extract. Carboxylation

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is implied in the conclusion by Steward, Bidwell, and Yemm (1956) that this amino acid is "en route to glutamic acid and glutamine". Amongst other evidence for this view Steward, Bidwell, and Yemm (1958) showed by feeding  $^{14}\text{C}$ -labelled ABA to explants of carrot root phloem that the carbon of the glutamine and glutamic acid being formed in the tissue was partly derived from the ABA. Warburg, Klotzsch, and Krippahl (1957) deduced from observations on *Chlorella* that an equilibrium between ABA and glutamic acid is maintained by the metabolic state of the cells. Fowden (1959) has suggested oxidative pathways as an alternative for the re-utilization of ABA.

As a contribution towards assessing the role of this amino acid, some results are reported here of a study, similar to the approach by Brown (1906), on the effect of externally applied ABA on nitrogen assimilation and growth of isolated germinating wheat embryos. The possibility was investigated that shoot-root ratios in the activity of some enzymes relevant to ABA metabolism could throw light on the utilization of ABA.

## II. MATERIALS AND METHODS

### (a) Feeding Experiments

Grains of *Triticum vulgare* L., cv. Nabawa, produced locally, were selected for uniform weight.

The grains were surface-sterilized, soaked for 2 hr, and laid out in sterile petri dishes on moistened filter paper for 20-24 hr at 25°C. The embryos with the primary root piercing the grain coats were excised with the scutellum attached and transferred to 1-in. diameter tubes containing 16 ml slanted agar medium. The embryos were orientated on the surface of the agar with the root pointing downwards. Each treatment consisted of 10 replicates. As the manipulation of 80 or more embryos lasted some hours, batches of explants containing all treatments were incubated at time intervals.

The basal medium was prepared by adding 8 g agar (Bacto-Difco) and 18 g glucose to 1 l. solution consisting of:

	Concn. (mm)		Concn. ( $\mu\text{M}$ )
KCl	0.2	MnCl <sub>2</sub>	2.0
CaCl <sub>2</sub>	0.4	H <sub>3</sub> BO <sub>3</sub>	1.0
MgSO <sub>4</sub>	0.2	ZnSO <sub>4</sub>	1.0
KH <sub>2</sub> PO <sub>4</sub>	0.2	Na <sub>2</sub> MoO <sub>4</sub>	0.2
K <sub>2</sub> HPO <sub>4</sub>	0.07	CuSO <sub>4</sub>	0.1
Fe (chelated with EDTA)	5.0 mg/l		

and of:

	Concn. (mg/l)		Concn. (mg/l)
Pyridoxal	1.0	Thiamine	0.1
Nicotinamide	0.1	Pantothenic acid	0.1
Folic acid	0.1	Riboflavin	0.1
<i>p</i> -Aminobenzoic acid	0.1	Biotin	0.01

TABLE I

EFFECT OF  $\gamma$ -AMINO BUTYRIC ACID ON TOTAL AND INSOLUBLE NITROGEN CONTENT AND GROWTH OF WHEAT EMBRYOS CULTURED IN DARKNESS  
 Mean values per seedling after 120 and 240 hr of incubation at 25°C in the dark. The grains from which the embryos were taken weighed 55.0-59.9 mg.  
 The designation "control" applies to embryos cultured on the basal medium, and " $\gamma$ -aminobutyric acid" to embryos cultured on the same medium to which  $\gamma$ -aminobutyric acid (6.0 mm) was added

Embryo Treatment	Period of Incubation (hr)	Total Nitrogen per Seedling ( $\mu\text{g}$ )		Insoluble Nitrogen per Seedling ( $\mu\text{g}$ )								Coleoptile Growth (mm)	First Leaf Growth (mm)	Root Growth	
		Shoots + Roots		Shoots + Roots		Shoots		Roots		Number	Total Length (mm)				
		Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.						
Control	0	117.1	3.4	98.1	1.8										
Control	120	120.3	1.3	104.1	3.3	68.6	2.1	35.6	1.8	46.8	50.5	4.0	147.3		
$\gamma$ -Aminobutyric acid	120	181.4	2.2	144.4*	3.1	95.3*	2.1	49.2*	1.9	48.7	64.0*	4.8	162.7		
Control	240	121.0		106.7	2.3	69.3	2.6	38.0	0.7	50.8	98.4	4.4	158.4		
$\gamma$ -Aminobutyric acid	240	222.5*		167.5*	5.3	118.2	4.1	49.7	5.0	48.7	163.3*	5.1	170.0		

\* This value differs significantly at least at the 1 per cent. level from the value recorded for control plants harvested at the same time.

Nitrogen sources (except glutamine) were added to the basal medium prior to heat sterilization at 110 lb/sq. in. for 10 min. ABA appeared stable under these conditions. Glutamine was added separately as a filter-sterilized solution to the heat-sterilized medium. The pH was c. 5.8.

In all experiments incubation of embryos was at 25°C in the dark, except for occasional inspection in orange light.

At harvest time the lengths of plant parts were measured and the plants stored in 70 per cent. alcohol. After several days the plants were boiled in fresh 70 per cent. alcohol and after a few days the alcohol was again renewed. The plants were washed twice with distilled water prior to determination of insoluble nitrogen content by the microKjeldahl method and Nesslerization of distillates. Where the insoluble nitrogen content of the shoot and the roots was estimated separately, the shoot included the scutellum.

### (b) Tests for Enzyme Activity

(i) *Glutamic Acid Decarboxylase*.—*Preparation of extracts*: Wheat grains were soaked and germinated on moistened filter paper for 2 or 3 days at 25°C in darkness. The seedlings were separated from the endosperm and divided into root and shoot parts. These parts were ground separately in a chilled mortar in the presence of 0.2M phosphate buffer, pH 5.3. The extracts were strained through muslin, made up to volume to the equivalent of 20 seedlings per ml with additional buffer, and then centrifuged for 10 min at 500 g and 1°C. The supernatant was tested for enzyme activity. *Manometry*: Enzyme activity was measured in duplicate by conventional Warburg technique at 30°C. The vessels contained 2.0 ml extract, 0.3 ml 0.1M glutamic acid (pH 5.3), 0.5 ml 1M sodium fluoride, 0.1 ml pyridoxal phosphate (approx. 50  $\mu$ g), and 0.1 ml water. Water replaced glutamic acid in the controls.

(ii) *Transaminase*.—*Principle*: Extracts were incubated with amino acid and  $\alpha$ -ketoglutarate, and the glutamic acid produced was assayed with bacterial glutamic acid decarboxylase (Cohen 1955). *Preparation of extracts*: Extracts of roots and shoots were prepared as for glutamic acid decarboxylase assay except that an 0.2M phosphate buffer, pH 7.5, was used. The extracts were dialysed overnight against 0.001M phosphate buffer, pH 7.5. In the interval the buffer was renewed once. On other occasions the extract was prepared with 0.15M pyrophosphate buffer, pH 8.5, and an ammonium sulphate precipitation (70 per cent. saturation) was carried out. The precipitate was redissolved in 0.015M pyrophosphate buffer, pH 8.5, to the original volume. *Procedure*: Into the main compartment of a Warburg vessel was pipetted 1.7 ml extract, 0.1 ml pyridoxal phosphate (approx. 50  $\mu$ g), 0.2 ml 0.5M amino acid dissolved in 0.01M buffer (either pH 7.5 or 8.5), and in the side-arm 0.2 ml 0.5M  $\alpha$ -ketoglutarate adjusted to pH. After incubation for 90 min at 30°C the reaction was stopped and the glutamic acid formed was assayed with bacterial glutamic acid decarboxylase (Worthington Biochem. Corp., U.S.A.).

(iii) *Amino Acid Oxidase*.—Oxygen consumption of extracts, either dialysed or redissolved from ammonium sulphate precipitates, was measured by the conventional



TABLE 2

EFFECT OF NITRATE AND DIFFERENT CONCENTRATIONS OF  $\gamma$ -AMINOBUTYRIC ACID ON INSOLUBLE NITROGEN CONTENT AND GROWTH OF WHEAT EMBRYOS CULTURED IN DARKNESS

Mean values per seedling after 120 hr of incubation at 25°C in the dark. The grains from which the embryos were excised weighed 55.0–59.9 mg. The mean value of the insoluble nitrogen content of the embryos at the time of excision was 98.3  $\mu$ g per embryo and the standard error 2.6  $\mu$ g

Substance Added	Concn. (mm)	Insoluble Nitrogen Content per Seedling ( $\mu$ g)		Coleoptile Growth (mm)	Root Growth	
		Mean	S.E.		First Leaf Growth (mm)	Total Length (mm)
Nil		95.2	2.6	53.8	49.0	137.8
$\gamma$ -Aminobutyric acid	3.0	112.1*	3.7	53.9	61.8*	138.7
$\gamma$ -Aminobutyric acid	6.0	133.1*	4.1	58.3	69.5*	132.3
$\gamma$ -Aminobutyric acid	12.0	137.0*	5.4	52.8	72.0*	108.2*
Potassium nitrate	3.0	221.0*	8.2	56.7	99.6*	300.3*

\* This value differs significantly at least at the 1 per cent. level from the value recorded for the treatment in which no substance was added to the basal medium.

Warburg technique. The central cup contained KOH, the main compartment 2 ml of extract, and the side-vessel 0.4 ml 0.05M amino acid.

### III. RESULTS

#### (a) *Feeding Experiments*

Table 1 summarizes the results of an experiment in which excised embryos were fed with ABA at 6.0 mM concentration and cultured for 120 and 240 hr. The insoluble nitrogen content of the ABA-treated seedlings is significantly higher than the total nitrogen content of the control embryos or of the zero-time embryos. This demonstrates that ABA nitrogen was utilized for protein synthesis. The growth in length of the first leaves was significantly stimulated by ABA, but the growth in length of the roots was not. This phenomenon was also observed in a series of experiments mentioned below. However, the insoluble nitrogen content of the roots of the ABA-treated seedlings was nearly 40 per cent. higher than that of the roots of the control seedlings. The roots virtually stopped growing after 120 hr of incubation, and their insoluble nitrogen content remained constant. In the shoots the insoluble nitrogen content continued to increase in the ABA-treated seedlings but at a reduced rate.

In another experiment in which embryos were cultured for 144 hr the roots of the control treatment attained an insoluble nitrogen content of 32.5 S.E.  $2.3 \mu\text{g}$  and a total length of 156.3 mm, and the corresponding values for the ABA treatment (3 mM) were 43.2 S.E.  $1.4 \mu\text{g}$  and 157.0 mm. The difference in insoluble nitrogen content was again significant but the difference in total root length was not.

Table 2 gives the results of an experiment in which different concentrations of ABA and potassium nitrate at 3.0 mM were applied to the embryos under conditions similar to those for the experiment of Table 1. All concentrations of ABA used effected a significant increase of insoluble nitrogen content. This increase approached its maximum value at 6.0 mM concentration. It is noteworthy that the seedlings appeared tolerant to a high concentration (12.0 mM) of the amino acid. The only adverse effect of this concentration was in total root length. At the other concentrations of ABA used, total root length was not increased over the control. Potassium nitrate surpassed the effects of ABA considerably in insoluble nitrogen content and in the lengths of the first leaf and of the roots.

In Table 3 the effects of ABA can be compared with those of the control, DL- $\alpha$ -aminobutyric acid, L- $\alpha$ , $\gamma$ -diaminobutyric acid, monosodium-L-glutamate, L-proline, L-alanine, L-asparagine, and L-glutamine. The amino acids were applied on a basis of nitrogen equivalence.

Only the control and the L- $\alpha$ , $\gamma$ -diaminobutyric acid treatments resulted in significantly less insoluble nitrogen content than the ABA treatment. The diamino acid proved very inhibitory to growth. It is noteworthy that  $\alpha$ -aminobutyric acid, although applied as a racemate, afforded as much nitrogen assimilation as ABA; except for a remarkable increase in root number, it had an inhibiting effect on growth. Glutamic acid, proline, alanine, asparagine, and glutamine gave significant increases over ABA in insoluble nitrogen content and growth. Root growth was stimulated

TABLE 3

EFFECT OF  $\gamma$ -AMINOBUTYRIC ACID AND SOME OTHER AMINO ACIDS ON INSOLUBLE NITROGEN CONTENT AND GROWTH OF WHEAT EMBRYOS CULTURED IN DARKNESS

Mean values per seedling after 120 hr of incubation at 25°C in the dark. The grains from which the embryos were excised weighed 60.0–64.9 mg. The mean value of the insoluble nitrogen content of the embryos at the time of excision was 109.5  $\mu$ g per embryo and the standard error 2.6  $\mu$ g

Substance Added	Concn. (mm)	Insoluble Nitrogen Content per Seedling ( $\mu$ g)		Coleoptile Growth (mm)	First Leaf Growth (mm)	Root Growth	
		Mean	S.E.			Number	Total Length (mm)
Nil		105.4	4.4	61.1	50.1	4.7	168.4
DL- $\alpha$ -Aminobutyric acid	3.0	123.1*	1.8	45.7*	33.7*	7.9*	60.1*
L- $\alpha$ , $\gamma$ -Diaminobutyric acid	1.5	103.2	3.1	4.9*	4.8*	3.6	16.2*
$\gamma$ -Aminobutyric acid	3.0	124.9*	2.8	61.9	57.2*	5.2	156.7
Monosodium L-glutamate	3.0	174.9*	0.7	61.8	73.0*	5.0	229.3*
L-Proline	3.0	158.0*	4.9	68.2	81.5*	5.2	146.4
L-Alanine	3.0	181.8*	9.4	64.4	80.1*	5.0	192.7*
L-Asparagine	1.5	207.8*	7.8	69.8	89.6*	4.9	244.0*
L-Glutamine	1.5	235.6*	10.4	68.9	94.7*	4.8	250.6*

\* This value differs significantly at least at the 1 per cent. level from the value recorded for the treatment in which no substance was added to the basal medium.

by glutamate but not by ABA and not by proline. There are no significant differences between the effects of asparagine and glutamine. Although Tables 2 and 3 are not strictly comparable, it is of interest to note that results with nitrate (Table 2) and with asparagine or glutamine (Table 3) are more or less equal.

(b) *Enzyme Tests*

(i) *Glutamic Acid Decarboxylase*.—Carbon dioxide evolution of root and shoot extracts of 2- and 3-day-old seedlings from whole grains incubated with glutamic acid was nearly linear for 80 min. The values observed for an incubation period of 60 min expressed per 40 seedlings and per mg insoluble nitrogen are recorded in Table 4. It is evident from these data that the roots show considerably more

TABLE 4

GLUTAMIC ACID DECARBOXYLASE IN SHOOTS AND ROOTS OF WHEAT SEEDLINGS

Carbon dioxide production by extracts from shoots and roots of plants germinated for 48 and 68 hr on water and on  $\gamma$ -aminobutyric acid (6.0 mM). The values represent differences found between manometric estimations in the presence and absence of glutamic acid at 30°C

Source of Extract	Carbon Dioxide ( $\mu$ l) Produced in 60 Min at 30°C			
	Per 40 Plants		Per Mg Insoluble Nitrogen of Extract	
	Shoot	Root	Shoot	Root
48-hr seedlings germinated in water	43	105	23	128
68-hr seedlings germinated in water	71	185	20	137
68-hr seedlings germinated in $\gamma$ -aminobutyric acid	77	204	27	162

enzyme activity than the shoots; on a protein basis, the activity is more than six times greater in the roots than in the shoots. Treatment of grains with ABA (6.0 mM) during soaking and germination had no effect on observed activities.

(ii) *Transaminase*.—Attempts to demonstrate the presence of ABA-glutamic acid transaminase in dialysed extracts of roots and shoots of 2- and 3-day-old seedlings from both whole grains and excised embryos were unsuccessful, although alanine-glutamic acid transaminase activity could readily be shown. Further, although Fowden (1959) reported ABA-glutamic acid transaminase to be inducible in yeast, treatment of grains or excised embryos with 6 mM ABA failed to yield seedlings with detectable activity of this enzyme. The enzyme would have been detected in such seedlings if it had had 2-3 per cent. of the activity of the alanine-glutamic acid transaminase. When extracts of ABA-treated seedlings were incubated at pH 8.5 with alanine and  $\alpha$ -ketoglutarate, glutamate formed, expressed as  $\mu$ l CO<sub>2</sub> evolved, was 356 per equivalent of 34 seedlings and 306 per mg insoluble nitrogen in extracts for shoot preparations, and 464 and 574 respectively for root preparations.



(iii) *Amino Acid Oxidase*.—Addition of 20  $\mu$ moles ABA to dialysed extracts from whole seedlings or to redissolved ammonium sulphate precipitates of crude extracts tested at pH 6.0, 7.5, and 8.5 did not stimulate oxygen consumption, the endogenous rate of which did not exceed 10  $\mu$ l per hour. Ascorbic acid was readily oxidized by such extracts tested at pH 6.0. The failure to find any activity is consistent with the work by Kenten and Mann (1952) who stated that extracts of barley seedlings did not possess any amine oxidase activity.

#### IV. DISCUSSION

In this period of rapidly increasing information on plant nitrogen metabolism it has become evident that it is difficult to generalize on the relative role of the different amino acids therein. ABA, once thought to form a side product, has been ranked as a central amino acid in *Endomycopsis* (Steiner 1959). Steward and co-workers (1956, 1958) have drawn attention to its possible role in higher plants. Barnes and Naylor (1959) made the remarkable observation that ABA stimulated the growth of excised pine roots much better than a series of amino acids including glutamic acid. Nitrate stimulated root growth better than ABA which indicates that in the other cases nitrogen was deficient. Apparently ABA is utilized readily as a source of nitrogen by pine roots.

The results recorded above for wheat demonstrate that ABA nitrogen is utilized in protein synthesis by this cereal. However, the utilization is slow when compared with nitrate or glutamine and other amino acids such as alanine, proline, and glutamic acid. The relative efficiency of various nitrogen compounds probably reflects the readiness with which a system takes up a substance and incorporates its nitrogen into glutamic acid. The central assignment of glutamic acid and glutamine has now been proved for yeast in physiological experiments by Folkes (1959) who showed that at least 87 per cent. of the nitrogen of ammonium sulphate passes primarily through this amino acid. The limiting factor in feeding glutamic acid itself is most likely to be found in its uptake; the fact that glutamic acid has strongly polar groups lends support to this interpretation. On this basis alanine and ABA might be expected to be taken up more rapidly than glutamic acid. The fairly good utilization of alanine is then further explained by the presence of highly active alanine-glutamic acid transaminase. In contrast, judging from its utilization, ABA nitrogen cannot readily be passed on to glutamic acid. The enzyme tests appear to support this conclusion.

Since ABA-glutamic acid transaminase was not detectable, the direct transfer of ABA nitrogen via such a transaminase reaction is probably not a significant pathway. Judging from the utilization of alanine and of ABA, ABA-glutamic acid transaminase should have had approximately 25 per cent. of the activity of the alanine-glutamic acid transaminase, but no trace of activity was found when extracts were tested at pH 7.5 or 8.5.

The shoot-root distribution of glutamic acid decarboxylase does not fit in with the relative increases in length and insoluble nitrogen content of these parts when fed with ABA. After 120 hr of incubation with ABA (Table 1) increase in

insoluble nitrogen content in roots stopped but continued in the shoots. It therefore seems unlikely that direct conversion of ABA to glutamic acid by a reversal of this enzyme reaction is a possible mechanism.

It seems probable from the results that ABA nitrogen is not passed on to glutamic acid in a single-step reaction in wheat. Furthermore, the participation of an ABA oxidase in a more indirect pathway is unlikely because of the negative results of the tests for this enzyme.

Another problem raised by this study concerns root growth because, although growth in length of roots is stimulated by glutamic acid, in general, it is not stimulated by ABA. Therefore, whilst stimulating protein synthesis in the root, ABA must effect a deviation from normal root development. Perhaps there is a connection between this fact and the observation by Barnes and Naylor (1959) that the ABA-treated pine roots contained a high number of dichotomous laterals.

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# THE SENSITIVITIES OF MIXED POPULATIONS OF BACTERIA TO INHIBITORS

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## Summary

A study was made concerning the effects of mixed populations of *Pseudomonas aeruginosa* and *Desulfovibrio desulfuricans* on the sensitivities of the individual species to three groups of inhibitors. It was found that the presence of *Ps. aeruginosa* produced in some cases an increase in resistance of *D. desulfuricans* to substituted phenols, a consistent increase in resistance of *D. desulfuricans* to nitroparaffins, and had no marked effect on their sensitivity to mercurials.

The presence of the sulphate-reducing bacteria produced a significant increase in resistance of the pseudomonads to some of the nitroparaffins, a consistent increase in resistance of the pseudomonads to mercurials, and a consistent increase in sensitivity of the pseudomonads to phenols.

The use of dead *Ps. aeruginosa* cells in the mixtures had no significant effect on the sensitivity of sulphate-reducing bacteria to phenols, mercurials, and nitroparaffins. The presence of dead sulphate-reducing bacteria produced an increase in resistance of the pseudomonads to mercurials and nitroparaffins but did not increase the sensitivity of the pseudomonads to phenols.

## I. INTRODUCTION

Numerous papers have been published concerning inhibitors that may be used to control microbial growth under different conditions. The common practice in an investigation of this type is to use a pure culture of the organism involved in the problem even though it is invariably accompanied by other species in nature. Observations in this Laboratory indicate that the concentrations of antibacterial agents sufficient to inhibit pure cultures are often different from those necessary to inhibit mixtures of the same species.

The objective of this investigation was to determine the sensitivities of mixed populations of *Pseudomonas aeruginosa* and *Desulfovibrio desulfuricans* to various antimicrobial agents. These organisms are commonly found together in several different types of deterioration problems and it was anticipated that the results might have some practical value in controlling these problems.

## II. MATERIALS AND METHODS

Pure cultures of *Ps. aeruginosa* and *D. desulfuricans* were grown in M-10-E medium (Morita and ZoBell 1955) buffered to pH 7.0 with phosphate buffer. The *Ps. aeruginosa* inocula were incubated at 30°C for 24 hr and the cultures of *D. desulfuricans* were incubated at the same temperature for 4 days. Sufficient cultures were prepared to inoculate all tubes in each experiment from the same suspension and the inocula were shaken vigorously before they were used to assure uniform suspensions.

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The inhibitors were added to three sets of duplicate screw-cap test tubes in concentrations ranging upwards from 50 p.p.m. in integrals of 50 p.p.m. Two sets of tubes were made up to 19 ml with M-10-E medium (pH 7.0) and autoclaved for 15 min at 15 lb/sq. in. pressure. One set was inoculated with 1 ml of *Ps. aeruginosa* and the other with 1 ml of *D. desulfuricans*. The third set was made up to 18 ml with M-10-E medium, autoclaved, and inoculated with 1 ml of each culture.

The tubes were incubated at 30°C for 2 days and at the end of this period, 0.5 ml of the medium in each tube was inoculated into 5 ml of fluid thioglycollate medium (Difco); these subcultures were incubated for 48 hr at 30°C. This procedure

TABLE 1  
SENSITIVITY OF MIXED POPULATIONS OF BACTERIA TO PHENOLS

Compound	Inhibitory Concentration (p.p.m.)			
	<i>Ps. aeruginosa</i>		<i>D. desulfuricans</i>	
	Pure Culture	Mixed Culture	Pure Culture	Mixed Culture
2,4,6-Trichlorophenol	975	700	50	100
<i>o</i> -Phenylphenol	1000	825	200	200
<i>p</i> -Nitrophenol	950	900	50	100
4-Chloro-2-nitrophenol	1000	700	50	100
2,4-Dinitrophenol	2000	1500	50	50

was necessary to determine the end-points for growth of *Ps. aeruginosa*. The end-points for growth of *D. desulfuricans* were determined by the production of hydrogen and iron sulphides. Several of the experiments were repeated to determine the reliability of the results.

When dead cells were used the procedure was the same as described above except that the cultures were autoclaved before they were used.

### III. RESULTS

An evaluation of the inhibitory activity of phenols against pure and mixed cultures of *Ps. aeruginosa* and *D. desulfuricans* was made (Table 1) and it was found that smaller amounts of the compounds were required to inhibit the mixtures than the pure cultures of *Ps. aeruginosa*. In the presence of the sulphate-reducing bacteria, the sensitivity of the pseudomonads increased 30 per cent. using 4-chloro-2-nitrophenol, 28 per cent. using 2,4,6-trichlorophenol, 25 per cent. using 2,4-dinitrophenol.



and 17 per cent. using *o*-phenylphenol. The 5 per cent. increase in sensitivity using *p*-nitrophenol was not significant. *D. desulfuricans* was more resistant to 2,4,6-trichlorophenol, *p*-nitrophenol, and 4-chloro-2-nitrophenol in the presence of the pseudomonads. The sensitivities of the sulphate-reducing bacteria to *o*-phenylphenol and 2,4-dinitrophenol were not affected in the presence of the pseudomonads.

Table 2 lists the results obtained when pure and mixed cultures were studied for their sensitivity to mercurials. The presence of *Ps. aeruginosa* did not have a significant effect on the sensitivity of *D. desulfuricans* to mercurials; however,

TABLE 2  
SENSITIVITY OF MIXED POPULATIONS OF BACTERIA TO MERCURIALS

Compound	Inhibitory Concentration (p.p.m.)			
	<i>Ps. aeruginosa</i>		<i>D. desulfuricans</i>	
	Pure Culture	Mixed Culture	Pure Culture	Mixed Culture
Phenylmercuri-8-hydroxyquinolate	100	800	125	150
<i>o</i> -Chloromercuriphenol	50	475	375	400
Phenylmercuric lactate	50	775	700	625
Phenylmercuric naphthenate	150	1500	1425	1275
Phenylmercuric acetate	50	575	400	400
Ethylmercurithiosalicylic acid	225	1375	425	350

the presence of sulphate-reducing bacteria resulted in a considerable increase in the concentrations necessary to inhibit *Ps. aeruginosa*. The increase ranged from sixfold in the case of ethylmercurithiosalicylic acid to 15-fold with phenylmercuric lactate.

A selected group of the new nitroparaffin inhibitors were studied and the results may be found in Table 3. The pseudomonads showed an increased resistance of 50 per cent. with 2-nitro-1-butanol, 40 per cent. with 2-nitro-2-ethyl-1,3-propanediol dipropionate, and 33 per cent. with 2-bromo-2-nitropropyl acetate in the presence of sulphate-reducing bacteria. The differences were not significant when 2-nitro-2-methyl-1-propanol, 2-nitro-2-ethyl-1,3-propanediol, and tris(hydroxymethyl)nitromethane were used.

The presence of pseudomonads increased the resistance of the sulphate-reducing bacteria to all of the nitroparaffins. The greatest increase noted was a fivefold effect with tris(hydroxymethyl)nitromethane.

Table 4 lists the results when dead cells were used in the mixtures. The presence of dead sulphate-reducing bacteria caused a fivefold increase in the resistance of pseudomonads to phenylmercuric lactate; however, the effect was not as pronounced as the 15-fold increase observed with living cells. It may be observed that living and dead pseudomonads increased the sensitivity of sulphate-reducing bacteria to the mercurial. The sensitivities of *Ps. aeruginosa* and *D. desulfuricans* were not affected by the presence of dead cells of either species when 2,4,6-trichlorophenol was used. The resistance of pseudomonads to 2-nitro-2-ethyl-1,3-propanediol

TABLE 3  
SENSITIVITY OF MIXED POPULATIONS OF BACTERIA TO NITROPARAFFINS

Compound	Inhibitory Concentration (p.p.m.)			
	<i>Ps. aeruginosa</i>		<i>D. desulfuricans</i>	
	Pure Culture	Mixed Culture	Pure Culture	Mixed Culture
2-Nitro-2-methyl-1-propanol	325	300	100	250
2-Nitro-1-butanol	300	450	50	200
2-Bromo-2-nitropropyl acetate	150	200	100	200
2-Nitro-2-ethyl-1,3-propanediol dipropionate	550	775	250	375
2-Nitro-2-ethyl-1,3-propanediol	400	350	100	225
Tris(hydroxymethyl)nitromethane	350	300	50	250

dipropionate increased 30 per cent. with dead cells as compared with 40 per cent. with live cells. The presence of dead pseudomonads had no effect on the sensitivity of the sulphate-reducing bacteria to the inhibitor.

Table 5 lists the effects of the addition of varying quantities of dead *D. desulfuricans* cells upon the sensitivity of pseudomonads to phenylmercuric lactate. It may be observed that as the quantity of dead cells increased the resistance of *Ps. aeruginosa* to the mercurial also increased.

#### IV. DISCUSSION

The effect of mixed populations of bacteria on the sensitivity of an individual species to an inhibitor is undoubtedly an important factor in the effective control of microbial growth. This fact seems to have been ignored as only one paper pertaining to this phenomenon has been found in the literature. Russell (1955) observed that

in certain instances deterioration of groundwood pulp could not be prevented by concentrations of phenylmercuric acetate that were normally inhibitory for the organisms causing the problem. He found that *Penicillium roqueforti* present in mercury-treated pulp was able to absorb relatively large quantities of mercury

TABLE 4  
EFFECT OF DEAD CELLS ON THE SENSITIVITY OF *PS. AERUGINOSA* AND *D. DESULFURICANS* TO INHIBITORS

Compound	Inhibitory Concentration (p.p.m.)			
	<i>Ps. aeruginosa</i>		<i>D. desulfuricans</i>	
	Pure Culture	1.0 ml of Dead <i>D. desulfuricans</i>	Pure Culture	1.0 ml of Dead <i>Ps. aeruginosa</i>
Phenylmercuric lactate	50	225	700	650
2,4,6-Trichlorophenol	1250	1250	50	50
2-Nitro-2-ethyl-1,3-propanediol dipropionate	500	650	100	100

from the environment. This organism reduced the active concentration of the inhibitor thereby permitting growth of *Stereum sanguinolentum*. This phenomenon resulted in the subsequent deterioration of the pulp.

TABLE 5  
EFFECTIVENESS OF PHENYLMERCURIC LACTATE AGAINST *PS. AERUGINOSA* IN THE PRESENCE OF DEAD *D. DESULFURICANS* CELLS

Amount of Dead Cells (ml)	Inhibitory Concentration (p.p.m.)	Amount of Dead Cells (ml)	Inhibitory Concentration (p.p.m.)
0.0	50	0.4	200
0.1	100	0.5	200
0.2	150	1.0	225
0.3	200	2.0	250

The results of the present investigation show that mixed populations of micro-organisms may have a significant effect upon the sensitivities of the individual species to inhibitors.

The effectiveness of an inhibitor is partly dependent upon the number of cells in the environment and in the present study twice as many cells were used in the mixture as in the pure cultures. Smyth (1934) found that doubling or halving the concentration of *Staphylococcus aureus* cells in the inoculum produced a 17 per cent. difference in the results of phenol death-rate determinations. Garrod (1935) demonstrated an even greater effect with the same organism when he observed that a concentration of 5000 p.p.m. of phenol was required to inhibit a large inoculum whereas a concentration of about 80 p.p.m. inhibited a small one. The quantity of dead cells is also important in determining the effectiveness of inhibitors. Tezuka (1940) found that dead cells take up more phenol than living cells. In the present investigation several experiments were done using 0.5 ml of each culture in the mixture instead of 1 ml and the same general trends were encountered with minor modifications. Additional proof may be found in the experiments with 2,4,6-trichlorophenol showing that the number of cells was not the factor involved in the change in sensitivity of the individual species. The mixture of viable cells (Table 1) had a marked effect on the sensitivities of the organisms whereas the same concentration of dead cells in the mixture (Table 4) had no effect on the sensitivities of the two species.

Additional experiments were carried out using different subcultivation media and this modification had no effect on the results. It appears that there is a significant difference in the sensitivities of two species to an inhibitor when they are in the same environment.

Many investigators have reported that bacteria can oxidize phenols (see Bennett 1959 for a short review of this topic) and these observations may account for the increased resistance of sulphate-reducing bacteria in the presence of pseudomonads to these inhibitors. These compounds were not completely inhibitory for *Ps. aeruginosa* in quantities that were effective against sulphate-reducing bacteria. It is possible that the pseudomonads reduced the environmental concentrations of the phenols by oxidation and as a result the sulphate-reducing bacteria were able to grow in the tubes initially containing higher concentrations of the inhibitors. An additional observation that may substantiate this observation is the fact that dead pseudomonads had no effect on the sensitivity of sulphate-reducing bacteria to these inhibitors.

The sensitivity of *Ps. aeruginosa* to phenols increased in the presence of living *D. desulfuricans* but not in the presence of dead cells. The presence of hydrogen sulphide in the inoculum of sulphate-reducing bacteria may have potentiated the inhibitory activities of the phenols. It is possible that the hydrogen sulphide concentrations were reduced when the cultures were autoclaved and the amounts left in the inoculum did not influence the inhibitory activities of the phenols against pseudomonads.

The results of the present investigation show that sulphate-reducing bacteria can have a profound effect on the sensitivity of pseudomonads to mercurials. Guynes and Bennett (1958) have reported that sulphate-reducing bacteria are not very sensitive to mercurials and that resistance may be due to their ability to produce hydrogen sulphide which precipitates the mercury ions. Hydrogen and iron sulphides could also protect the pseudomonads from the effects of mercurials if sulphate-reducing bacteria are present in the environment. It is of interest to note that in



four of the experiments the presence of sulphate-reducing bacteria increased the tolerated levels of mercurials for pseudomonads to those that were inhibitory for *D. desulfuricans*. Further proof that sulphides may be involved can be obtained from the studies with dead cells. The presence of dead sulphate-reducing bacteria had a significant effect on the sensitivity of pseudomonads to these inhibitors.

It is difficult to explain why the mixture had an effect on the sensitivities of *Ps. aeruginosa* and *D. desulfuricans* to the nitroparaffin derivatives. There are only two reports pertaining to the inhibitory activity of these compounds against bacteria (Urbanski 1951; Wheeler and Bennett 1956) and nothing is known regarding the manner in which they inhibit bacteria.

In the presence of 2-nitro-2-ethyl-1,3-propanediol dipropionate, the dead sulphate-reducing bacteria were about as effective as living cells in increasing the resistance of pseudomonads to this inhibitor. On the other hand, the presence of dead pseudomonads had no effect on the sensitivity of the sulphate-reducing bacteria whereas living cells produced a 50 per cent. increase in the resistance of the sulphate-reducing bacteria to this inhibitor. The results indicate that there are at least two different mechanisms involved.

Sulphate-reducing bacteria may produce some extracellular product that influences the sensitivity of pseudomonads to these compounds. It has been reported that culture filtrates of *Ps. aeruginosa* can antagonize the action of streptomycin, dihydrostreptomycin, and neomycin (Lightbown 1950; Bergman *et al.* 1954). There is a possibility that a similar phenomenon occurred in the present investigation with sulphate-reducing bacteria.

There is another possible explanation that may be found in some observations that have been made with phenols. Gale and Taylor (1947), Pulvertaft and Lumb (1948), Yanagita and Suzuki (1948), Stedman, Kravitz, and King (1957), and Beckett, Patki, and Robinson (1958) have observed that phenols cause a leakage of intracellular components from bacteria. Bean and Walters (1955) have suggested that the release of these constituents may influence the viability of the last survivors and alter the course of the killing reaction. The nitroparaffins may have caused a leakage of materials from the sulphate-reducing bacteria that affected the sensitivity of the pseudomonads. It is inconceivable that the sulphate-reducing bacteria oxidized the nitroparaffin derivatives in this particular instance because in almost every case the concentrations were higher than those required to inhibit these organisms.

It is possible that the pseudomonads oxidized the nitroparaffins to a limited extent and thus increased the concentrations that the sulphate-reducing bacteria could survive in. This may account for the fact that dead pseudomonad cells had no effect on the sensitivity of the sulphate-reducing bacteria. On the other hand, this phenomenon may be due to production of thermolabile compounds by the pseudomonads.

#### V. ACKNOWLEDGMENT

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# SIMULATION OF GENETIC SYSTEMS BY AUTOMATIC DIGITAL COMPUTERS

## VI. EPISTASIS

By A. S. FRASER\*

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### *Summary*

Simulation, by Monte Carlo methods, of the effect on the genotype of selection against phenotypic extremes has shown that selection will lead to fixation of a simple additive genetic system at an extremely slow rate in all but very small populations. In complex epistatic systems, such selection operates to modify the relation of the genotype to the phenotype. The relationship becomes an S-shaped function. The efficiency of selection is independent of population size. The deviation from initial gene frequencies due to selection is far less per unit decrease of phenotypic variability in the epistatic than in the additive lines.

## I. INTRODUCTION

The work of this Laboratory on quantitative inheritance has recently centred on the problems posed by the existence of genetic variability in the absence of phenotypic variability (see Rendel 1959; Fraser and Kindred 1960). Consequently, the work with simulation programmes on electronic computers has been directed at the problem of selection against extreme phenotypes. Robertson (1956), in a theoretical analysis of this problem, examined two alternatives: the first, in which extremes are less fit because they are extremes, he found would lead to fixation; the second, in which extremes are less fit not because they are extremes but because they are homozygotes, he found would maintain genetic variability. He, thus, clearly supports Lerner's thesis that heterozygotes have an enhanced fitness *per se*, i.e. that there is overdominance of fitness (see Lerner 1958). Robertson did not consider the effect of such selection against extreme deviants on epistatic interactions, yet it seems probable that selection can modify such interactions and, consequently, reduce the trend towards homozygosity (Rendel 1959). A programme written to simulate a genetic system in which both the dominance and epistatic relations were under genetic control indicated that fixation of the basic loci will be considerably reduced by modification of epistatic relations (Fraser 1959). There were, however, several features of this programme which might produce bias, the most important being an inherent asymmetry. In addition, the programme was, by computer standards, slow, and it was limited to a maximum population size of slightly less than 200 progeny. A new programme has been written which, although based on a larger number of loci—40 as against 20—is faster and puts no limit on the number of progeny per generation, the limit being on the number of parents. This programme is described below, and results from two sets of its runs discussed.

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## II. STRUCTURE OF THE EPISTASIS PROGRAMME

The programme has six main sections:

- (1) Extract, without replacement, a pair of parents at random from the given set of parents.
- (2) Form a set of progeny from these parents.
- (3) Determine the phenotypes of the progeny.
- (4) Select potential parents from the set of progeny.
- (5) Repeat (1)–(4) until all parents have produced the specified number of progeny.
- (6) Print out any required information.
- (7) Repeat (1)–(6) using the selected progeny as parents.

## III. SIMULATION OF SEGREGATION

Section II includes the simulation of segregation. The method initially described by Fraser (1957*a*, 1957*b*) is based on a "random walk" along the length of the genotype, each locus being considered separately. Mr. J. B. Butcher, Adolph Basser Computing Laboratory, has suggested a method which obviates considering each locus in sequence, and makes full use of the parallel arithmetic of the SILLIAC computer.

A single SILLIAC register contains 40 "bits" of binary information, and orders in the SILLIAC suppressing carry-over allow various operations of "logical algebra" to be performed separately, and simultaneously, for all 40 bits of a register. From such operations it is possible to identify the genetic status of 40 loci in a single process. Given such identification it is then easy to simulate segregation simultaneously at 40 loci.

This is accomplished by the following sequence, given that register A contains the "maternal genotype" and register B the "paternal genotype" of a parent which is to produce gametes:

- (1) Form the logical product (L.P.) of A and B

1	1	0	.	.	.	.	.	A
1	0	0	.	.	.	.	.	B
<hr/>								
1	0	0	.	.	.	.	.	L.P. (A/B)

- (2) Form the logical non-equivalent (L.N.E.) of A and B

1	1	0	.	.	.	.	.	A
1	0	0	.	.	.	.	.	B
<hr/>								
0	1	0	.	.	.	.	.	L.N.E. (A/B)

The L.P. (A/B) identifies the loci homozygous for the 1 type alleles; the L.N.E. (A/B) identifies the heterozygous loci.



(3) Form the L.P. of a random number (C) with the L.N.E. (A/B)

0	1	0	.	.	.	.	.	L.N.E. (A/B)
0/1	0/1	0/1	.	.	.	.	.	C

---

0	0/1	0	.	.	.	.	L.P. (L.N.E. (A/B)/C)
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(4) Add the L.P. (A/B) to the L.P. (L.N.E. (A/B)/C)

0	0/1	0	.	.	.	.	L.P. (L.N.E. (A/B)/C)
1	0	0	.	.	.	.	L.P. (A/B)

---

1	0/1	0	.	.	.	.	Simulated gamete
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As a result of these operations 1 is placed in every position representing a gene of the 1 type which was homozygous in the parent and 0 in every position representing a homozygous gene of the 0 type; the heterozygous genes are represented by 1 or 0 at random.

These operations can be performed simultaneously for up to 40 digits. Apart from the formation of a random number, this sequence takes seven orders, totalling 395  $\mu\text{sec}$ .

#### IV. DETERMINATION OF THE PHENOTYPE

The genetic model is of 40 independent loci. The phenotype determined by these loci has two components,  $P_0$  and  $P_1$ , each determined similarly by 20 loci. Each group of 20 loci is considered as four sets of five loci. The relationships of these to the phenotype are the same as those in the initial epistasis programme (Fraser 1959). These four subgenotypes are termed the  $A$ ,  $D$ ,  $Q$ , and  $C$  genotypes respectively.

The  $A$  group of five loci determines a contribution to the phenotype which in the absence of dominance or epistasis is given for each locus by

Genetic Status	Phenotypic Contribution
$\frac{1}{1}$	-1.0
$\frac{1}{0}$	0
$\frac{0}{0}$	+1.0

The "additive" contribution of the  $A$  subgenotype to the phenotype is given by summation over the five loci.

The  $D$  group of five loci determines the degree of dominance at the loci of the  $A$  genotype. This genotype simulates a dominance modifier system affecting identically the five loci of the  $A$  genotype. It determines the phenotypic contribution of heterozygotes of the  $A$  group, and has no effect on the phenotypic contribution of the homozygotes.

The  $D$  loci do not themselves have any dominance, i.e. over the five loci of this genotype there are 11 possible values conferring dominance on the  $A$  group. A vector of possible dominance values is specified for a particular run, and remains constant for that run. The value of the  $D$  genotype in an individual determines which value of this vector is appropriate.

If the vector of possible dominance coefficients is  $\{d_i\}$ , then:

Genetic Status	Phenotypic Contribution
$\frac{1}{1}$	$-1.0$
$\frac{1}{0}$	$d_i$
$\frac{0}{0}$	$+1.0$

The vector  $\{d_i\}$  has been set, for the runs which are discussed in this paper, at either zero, or in a linear sequence covering the range  $+1.0 \leq d_i \leq -1.0$ , such that a substitution in the  $D$  genotype of a 0 for a 1 type allele makes a difference of 0.2 in  $d_i$ , which gives a variation from complete dominance of the 1 alleles ( $d_i = -1.0$ ) through no dominance ( $d_i = 0.0$ ) to complete dominance of the 0 alleles ( $d_i = +1.0$ ).

Summation over the five loci of the  $A$  genotype then gives the additive + dominance contribution to the phenotype. This is termed  $(P_a + P_d)_0$ .

The degree of interaction between the five loci of the  $A$  genotype is determined by the  $Q$  and  $C$  genotypes. Just as a particular  $D$  genotype determines a specific value of  $d_i$ , so the  $Q$  and  $C$  genotypes determine specific values of quadratic and cubic interaction coefficients from the vectors of such coefficients:  $\{q_i\}$  and  $\{c_i\}$ . These have been set for the runs which are discussed in this paper at either zero, or in linear sequence covering the following ranges:

$$+0.5 \leq q_i \leq -0.5; \quad +0.125 \leq c_i \leq -0.125.$$

The coefficients  $q_i$  and  $c_i$ , determined by specific  $Q$  and  $C$  genotypes, give the degree of interaction by

$$P_Q = (P_a + P_d)_0^2 q_i,$$

and

$$P_C = (P_a + P_d)_0^3 c_i.$$

The complete determination of the  $P_0$  component of the phenotype is given by

$$P_0 + (P_a + P_d)_0 + q_i(P_a + P_d)_0^2 + c_i(P_a + P_d)_0^3.$$

The other 20 loci similarly determine  $P_1$ , and the total phenotype is the simple sum of  $P_0$  and  $P_1$ .

The subdivision of the phenotype into two independently determined components was introduced to allow independent variation of the dominance genotypes. Mather (1943) has suggested that the lack of dominance in a quantitative system

may be due to individual loci of the system having dominance values in opposite directions. The average value of dominance over all the loci can then be zero, and variation still have a dominance component. In our model, two values of dominance are possible, one for each set of  $A$  loci, and it was originally considered that this could provide data on the feasibility of Mather's suggestion. Latter (personal communication) has made the point that each  $D$  genotype sets a dominance value for five  $A$  loci, and, consequently, any trends towards different dominance values between these loci would average out to zero. An adequate test of Mather's hypothesis requires an individual dominance modifier system for each  $A$  locus. The separation of the phenotype into two components is therefore only of value in indicating the consistency of any trends.

## V. METHOD OF SELECTION

In this programme, each progeny as it is formed is tested against fixed phenotypic limits which are specified at the beginning of the run, and remain constant for the duration of the run. Since the number of individuals required as parents ( $n$ ) may be less than the number of individuals with phenotypes within limits, the first  $n$  acceptable parents are provisionally accepted and placed in the "parent" store. Thereafter each acceptable parent is tested for substitution in that store against a random number, with a probability of  $n/i$ , where  $i$  is the total number of acceptable parents which have so far been tested. This ensures that all potential parents have an equal probability of being accepted as parents. The selection limits set for the runs discussed in this paper are  $\pm 1.0$ , i.e. the effect of a single gene substitution on the simple additive scale.

This method of simulating selection, although fast, has the disadvantage that the selection limits cannot be easily varied during a run. Another method, which will be used in a modification of this programme, does not have this disadvantage. It is based on the sequence of pseudo-random numbers being completely determined. Given the values of the random numbers at a specific point in a run, it is possible, by substituting these values, to repeat any particular sequence of the operations of the programme. This ability to repeat a sequence can be used to determine selection limits. At the beginning of the formation of progeny the values of the random number generator are stored. Then all, or a sample, of the progeny are formed and their phenotypes computed. Only sufficient information is retained to describe the frequency distribution of phenotypes. From this, selection limits are computed which will contain a specified fraction of the progeny. Given such limits, the original values of the random number generator are substituted, and the generation of progeny restarted. Each progeny as formed is then tested for acceptance against the selection limits.

## VI. PRE-SET PARAMETERS

Although the "epistasis" programme has been constructed to minimize the number of parameters initially specified, and thereafter, maintained constant, some must be specified and these are:

- (1) Whether random mating or self-fertilization is to occur.
- (2) The number of parents.
- (3) The number of progeny per mating.
- (4) The maximum and minimum limits of eligible phenotypes.
- (5) The identification number of the run.
- (6) The vectors of dominance and interaction coefficients.
- (7) The genotypes of the initial set of parents.

Sixteen runs of the programme have been made: eight in the absence of any dominance or epistasis, and eight in the presence of such effects. These runs were made at four population sizes: 20, 40, 80, and 160 parents, two runs at each population size. The number of progeny per mating was set at 50 for all runs, making the total numbers of progeny 500, 1000, 2000, and 4000. Two independent runs were made for each set of parameters.

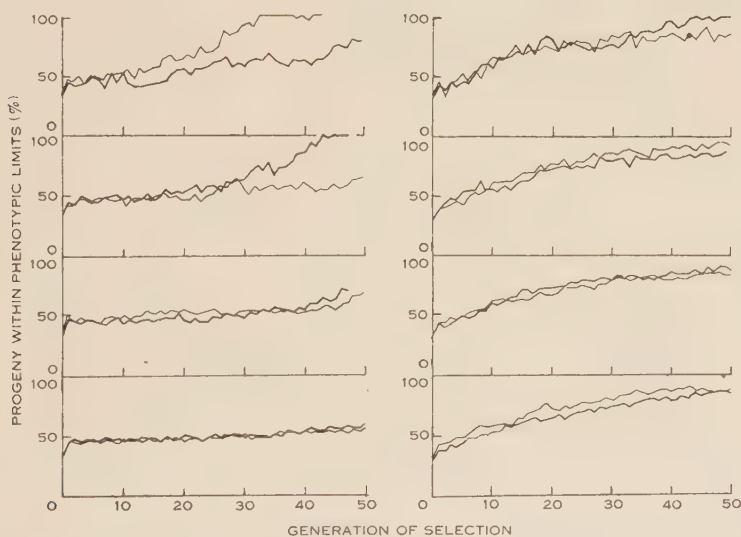


Fig. 1.—Percentage of progeny within the phenotypic limits plotted against generation of selection. Runs made in the absence of dominance and epistasis on the left. Runs made in the presence of dominance and epistasis on the right.

The absence of dominance and epistasis was produced in the first eight runs by setting  $\{d_i\}$ ,  $\{q_i\}$ , and  $\{c_i\}$  at zero. These genotypes consequently have no effect on the phenotype, and selection is directed solely at the  $A$  genotype, i.e. selection is operating on a simple additive system of 10 loci. The  $D$ ,  $Q$ , and  $C$  loci, totalling 30, are, in these runs, not under selection and changes of their frequencies can be taken as the basis for comparison with the  $A$  loci to determine the changes produced by selection.

All parents at the beginning of all runs were heterozygous at all loci.



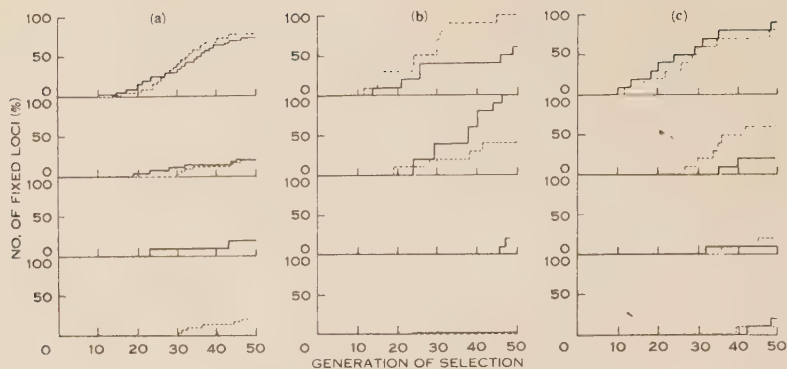


Fig. 2.—(a) Genetic fixation in the absence of selection. The percentage of *D*, *Q*, and *C* loci which have become fixed is plotted against generation for the eight runs in which these loci have no phenotypic effect. (b) Genetic fixation of a simple additive system. The percentage of *A* loci which have become fixed, plotted against generation of selection for the first eight runs. (c) Genetic fixation of the *A* loci in runs which include selection for modifiers of dominance and epistasis.

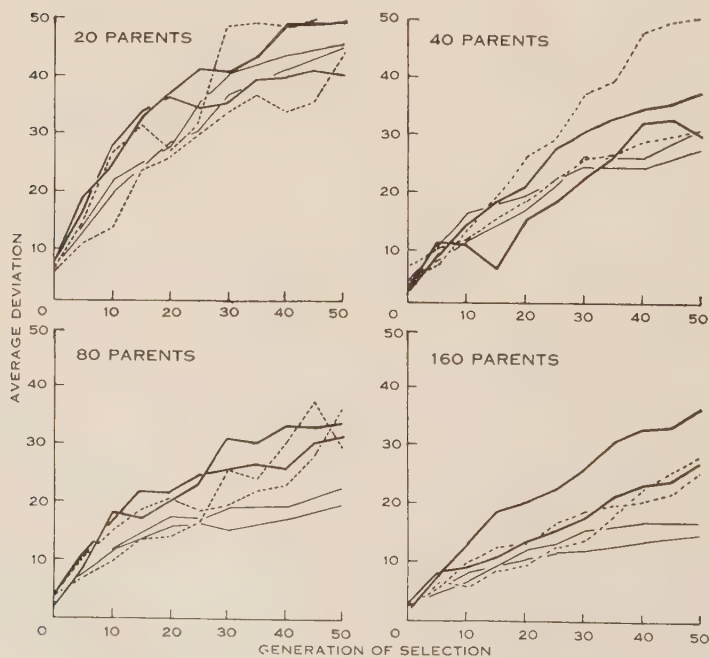


Fig. 3.—Average deviation of the frequencies of the *A* loci from the initial value of 0.5 is shown plotted against generation for (a) unselected loci (---); (b) simple additive system (—); (c) epistatic system (—).

## VII. RESULTS AND DISCUSSION

*(a) Phenotypic Variability*

The percentage of progeny produced each generation which have phenotypes within the specified phenotypic limits is a measure of the effectiveness of selection against the extreme phenotypes. These percentages are shown plotted against generation of selection in Figure 1.

In considering the eight runs made in the absence of dominance and epistasis, there is a marked effect of population size. In small populations, selection causes,

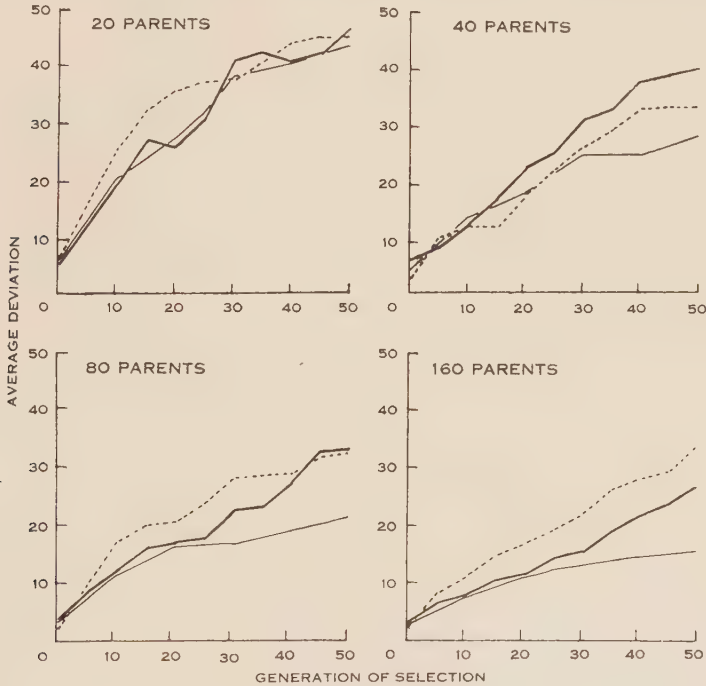


Fig. 4.—As for Figure 3, but the two replicate runs have been averaged.

at the small population sizes, a marked increase of the percentage of progeny with phenotypes within the selection limits. In large populations the percentage of acceptable progeny increases more slowly. This contrasts very markedly with the eight runs made in the presence of dominance and epistasis; the percentage of "acceptable" progeny increases rapidly over the initial generations of selection, and then more slowly as selection proceeds. The effect of population size is slight, being most apparent in a lower variation from generation to generation in larger populations (see Fig. 1).

The effect of selection on phenotypic variability in the two sets of populations is sufficiently different to suggest that different mechanisms are operating. This is shown by the genotypes which the machine recorded each generation.

The effect of selection and population size on the distribution of gene frequencies is shown in Figure 2 in which the percentage of loci which have become fixed is plotted against generation of selection and in Figure 3 in which the average deviation of gene frequencies from the initial value of 0.5 is shown plotted against generation of selection.

The incidence of genetic fixation shows that there is a marked effect of population size and that there is little if any effect of selection. The deviations from the original gene frequency show similarly that there is a marked effect of population size. Selection is ineffective in smaller populations of 20 and 40 parents but in ones

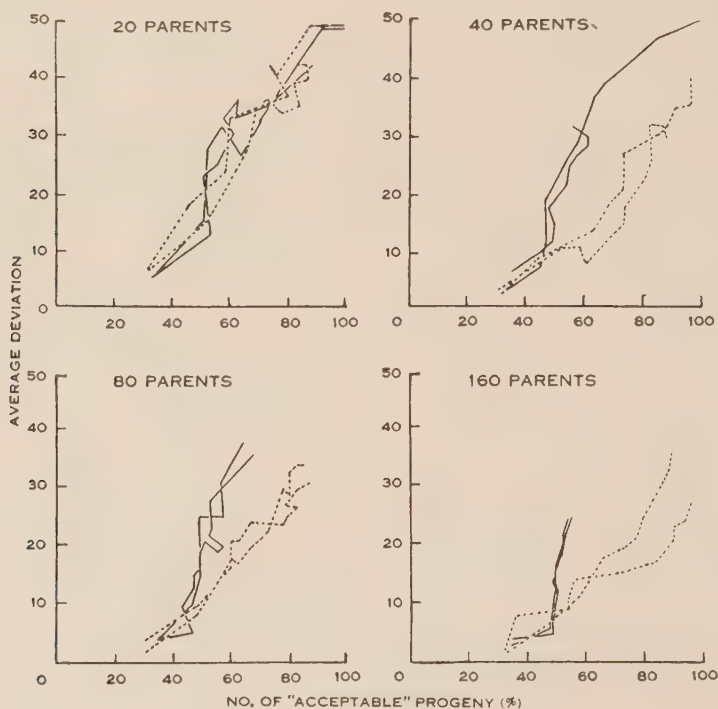


Fig. 5.—Average deviation of the frequencies of the *A* loci from initial value of 0.5 plotted against the percentage of "acceptable" progeny.  
— Additive system; ---- epistatic system.

of 80 and markedly in ones of 160 parents there is a real difference between the changes of gene frequency of unselected and selected loci. This is shown more clearly in Figure 4, in which the two replicate runs have been averaged.

Figure 4 indicates that selection of an epistatic system produces a greater change of gene frequency than selection of a simple additive system. However, no account has been taken of the effectiveness of selection in reducing phenotypic variability. This is particularly evident in large populations. In large populations without dominance or epistasis, i.e. a simple additive system, selection for over 50 generations has produced only a slight reduction of phenotypic variability whereas with dominance or epistasis, i.e. for the epistatic system, selection has

produced a very marked reduction of variability. Consequently, the percentage of progeny within limits is shown plotted against deviations from the initial gene frequency in Figure 5. These graphs show that selection against extreme phenotypes produces a much greater reduction of variability for a specified deviation from the initial gene frequency in the "epistatic" runs than in the "additive" runs.

The dominance and epistasis runs were made with  $\{d_i\}$ ,  $\{q_i\}$ , and  $\{c_i\}$  set to the ranges specified in Section IV. Variation of the  $D$ ,  $Q$ , and  $C$  genotypes due to segregation will therefore produce variation of  $d_i$ ,  $q_i$ , and  $c_i$ . This will affect  $P_i$  and consequently selection can affect the  $D$ ,  $Q$ , and  $C$  genotypes. The mean values

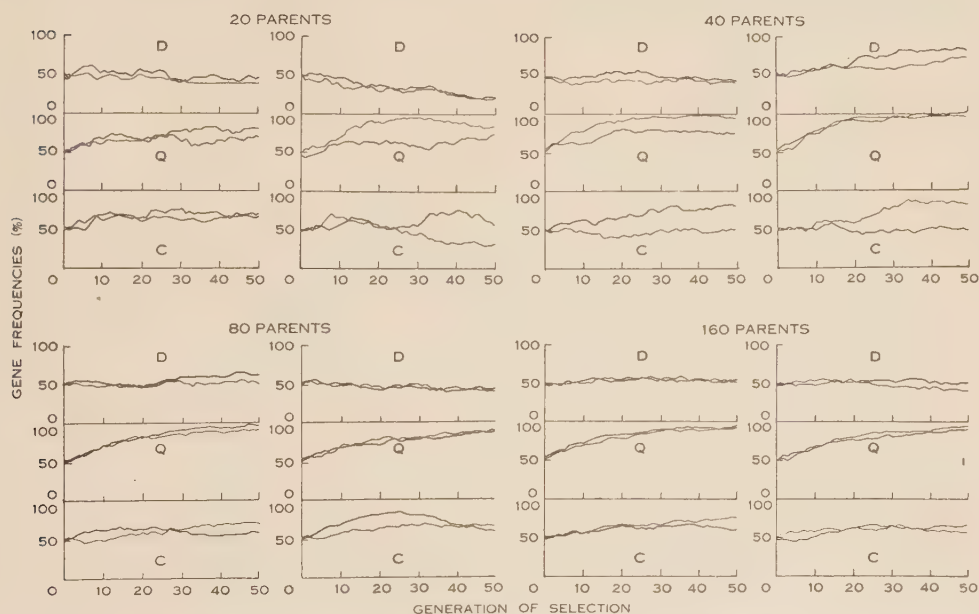


Fig. 6.—Average gene frequencies of the loci of the  $D$ ,  $Q$ , and  $C$  subgenotypes, shown separately for the four population sizes. There are two  $D$ ,  $Q$ , and  $C$  subgenotypes for each run, and two runs were made at each population size.

of the gene frequencies of the  $D$ ,  $Q$ , and  $C$  genotypes are given in Figure 6, plotted against generation of selection for the two runs made at each of the four sizes of population.

The dominance genotypes show no consistent changes from the original frequencies except in small populations where such deviation are probably due to random sampling effects. The effect of selection on the percentage of progeny with phenotype within limits cannot be ascribed to modification of the dominance system.

The epistasis genotypes show consistent changes such that the relationship of genotype to phenotype deviates considerably from a linear function. In the runs at smaller population sizes the high incidence of genetic fixation interacts with the selection for epistasis. This is not evident at larger population sizes. There is a trend towards a gene frequency of  $0.8 \rightleftharpoons 0.9$  in the  $Q$  genotypes and of



$0.5 \rightleftharpoons 0.7$  in the  $C$  genotypes, which correspond to a range of  $-(0.3 \rightleftharpoons 0.4)$  for  $q_i$  and of  $0.0 \rightleftharpoons -0.5$  for  $c_i$ . These ranges of  $q_i$  and  $c_i$  correspond to a set of curves of the type shown in Figure 7.

Clearly the increased percentage of individuals with phenotypes within the selection limits has been produced by changes in frequency of the  $Q$  and  $C$  genotypes; these have brought about a relationship of phenotype to genotype such that the majority of  $A$  genotypes have phenotypes within the selection limits of  $\pm 1.0$ .

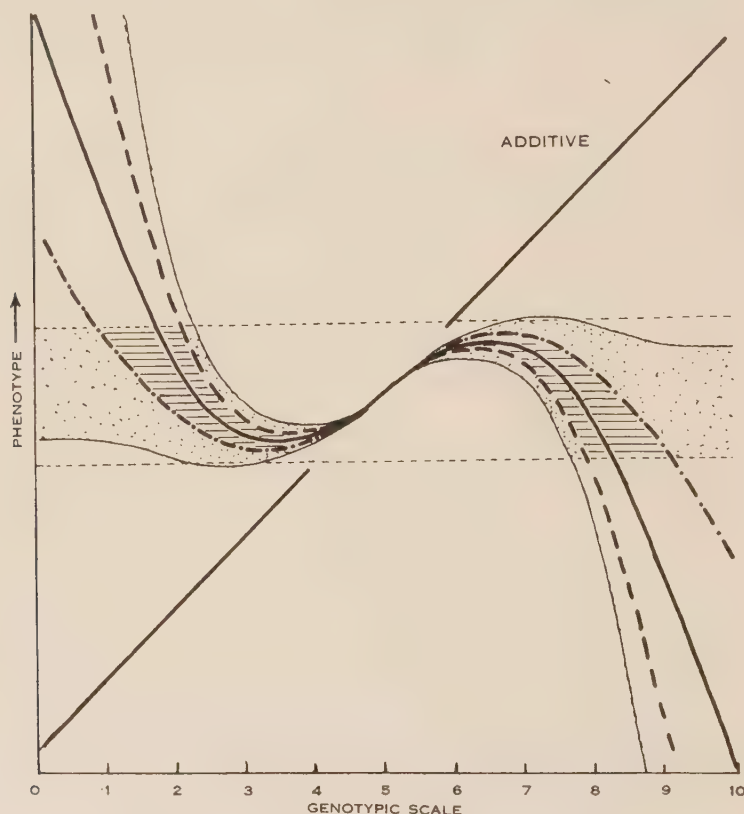


Fig. 7.—Relationship of genotype to phenotype for  $q_i = 0.4$  and  $c_i = +0.025, 0.0, -0.025, -0.05, -0.075$ .

### (b) Conclusion

The runs made with this programme have shown that selection of an additive genetic system will in the absence of epistasis lead to an increased degree of genetic fixation, as shown by the straightforward mathematical analysis (see Robertson 1956). This is especially marked in runs made at small population sizes. Inclusion of a variable degree of epistasis, under genetic control, causes a marked reduction of the rate of genetic fixation for any given degree of phenotypic uniformity.

Selection of the epistasis genotypes causes a change of the genotype-phenotype relationship from the linear, additive form, to a complex form which is such that

the majority of the  $A$  genotypes have the same phenotype. The effect of this on the frequency distribution of phenotype is shown in Figure 8.

Selection against extreme phenotypes can, in our genetic model, affect either the genetic system determining the genotype-phenotype relationship of the  $A$  loci or the degree of fixation of the  $A$  loci. It is clear that the former effect predominates. Some phenotypic variability still does occur even after 50 or more generations of selection. This is due predominantly to segregation of the  $Q$  and  $C$  genotypes, rather than of the  $A$  genotypes. Consequently, long continued selection should, eventually, result in the fixation of the epistasis genotypes. Selection of a complex genetic system, although decreasing the rate of fixation of the additive loci, results in fixation of the epistasis-determining loci, i.e. selection against extremes results in fixation, but in a complex system this fixation is restricted to a specific part of the genotype.

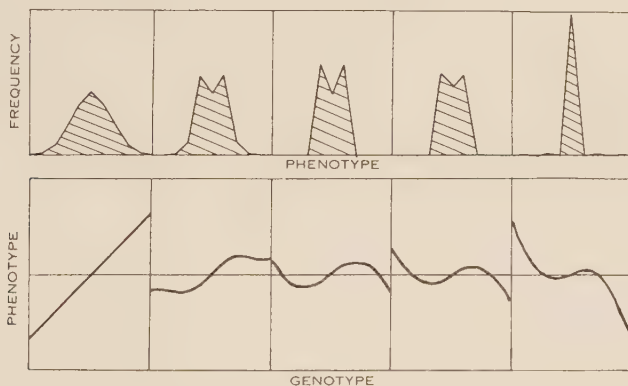


Fig. 8.—Frequency distribution of phenotypes when the frequency distribution of genotypes corresponds to an  $F_2$  from completely heterozygous parents. This is shown for several genotype-phenotype relationships.

An intriguing feature of these runs has been the form of the genotype-phenotype relationship which has been produced. Such a relationship, if genetically fixed, will have unexpected effects on the progress of selection towards an extreme phenotype. Suppose that selection against extreme phenotypes has caused the fixation of such a relationship, and that the gene frequencies of the additive loci are distributed to form a normal distribution centred on a "zero" phenotype. Then selection towards either an extreme positive or an extreme negative phenotype will initially cause a straightforward shift of the distribution. However, as selection proceeds the distribution will be moved towards the inflexion point of the genotype-phenotype relationship, and this will produce a marked decrease of the phenotypic variance, due to an increase in the proportion of genotypes having the same phenotype. The phenotypic distribution will become skewed against a selection limit, and further selection for the extreme phenotype will be against the genetic extremes, resulting in fixation of the additive loci. It would seem that no further advance could be achieved. However, if the extreme genotypes of the positive-selected

population could be identified, they could act as a basis for a negative selection line in which there would be no epistasis restrictions on the effectiveness of selection.

The examination of epistatic relationships has rarely been taken past the identification of a term in the analysis of variance. This is due to the attitude that selection can only act on additive variance. If the more general view is adopted—that epistatic systems can, as has been shown for dominance systems, be affected by selection—then it is pertinent not only to determine the existence of epistasis, but also to measure the pattern of its effect. The results of Waddington (1957), Dun and Fraser (1959), and Rendel and Sheldon (1960) have demonstrated that knowledge of the pattern of epistasis is useful in the design and interpretation of artificial selection experiments. Many of the ambiguities found in selection experiments when selection pressures are reduced or reversed suggest that mechanisms of this type may operate and are certainly worth considering as a basis for experiment.

Simulation of genetic systems by programming an electronic computer is a research activity readily available to the experimenter for the small expenditure of time taken to learn the techniques of programming. As a concomitant to actual experiments it will allow the gap between the theoretical and experimental geneticists to be bridged almost effortlessly. Genetic models can be devised, programmed, and tested within weeks or months; certainly with sufficient speed for an experimenter to examine many of the theoretical consequences of his ideas before he devises experiments with live organisms. This would seem to be the field in which this method can be used to the greatest value, though its use in the methodical examination of the importance of variables determining the effectiveness of selection is undeniable. However, it is more economical to restrict such extensive studies to programmes for extremely fast machines with very large memories allowing maximum efficiency, a very necessary feature when time on a machine may total several hundred hours.

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# PATHOLOGY OF INFESTATION OF THE RAT WITH *NIPPOSTRONGYLUS MURIS* (YOKOGAWA)

## II. CHEMICAL CONSTITUENTS OF THE JEJUNUM AND DRY WEIGHT OF THE MUCOSA

By L. E. A. SYMONS\*

[Manuscript received November 6, 1959]

### *Summary*

There was a 50 per cent. gain in the dry weight of the mucosa relative to the unit length of the jejunum of rats infested by the nematode *Nippostrongylus muris*. The importance of this to measurements of the rate of intestinal fluxes is discussed.

The concentrations of the electrolytes sodium and chloride were increased in total jejunal tissue on both a wet weight and a fat-free dry weight basis. Potassium concentration increased on the dry weight basis but was unchanged in wet tissue. The water content of this tissue was also greater in the infested rats but the fat content fell. There was a twofold increase in the volume of whole blood in the jejunum. The physiological significance of these changes are not clear but their possible association with undernourishment is suggested.

### I. INTRODUCTION

In Part I of this series (Symons 1957) it was reported that the dry weight of the tissues of the small intestine of the rat infested by the nematode *Nippostrongylus muris* (Yokogawa, 1920) was increased by about 50 per cent. In histological sections there was a twofold increase in the thickness of the muscularis externa which appeared to be hypertrophied. Although no quantitative measurements were made it also appeared that the bulk of the mucosa was increased. Not all of the latter could be accounted for by the oedema of the villi.

The hypertrophy of the muscularis externa and retention of ingesta in the infested jejunum (Symons 1959) were evidence of changes in the motor function of the gut. Black (1955) has stated that potassium depletion has been demonstrated in a number of alimentary disorders, including gastro-enteritis. Severe potassium depletion can cause intestinal dilatation and even paralytic ileus (Schlesinger, Payne, and Black 1955). These changes are believed by Streeten and Vaughan Williams (1952) to be due to a loss of intracellular potassium, although their dogs depleted of sodium and chloride had elevated plasma potassium levels. Daniel and Bass (1956), who investigated the effect of electrolyte depletion upon gastro-intestinal motility of the rat, concluded that the tract resisted potassium depletion and that the mechanism controlling its electrolyte distribution probably differed from that in skeletal muscle. They did, however, show that electrolyte deficiencies could be reflected in the gut and may decrease propulsion. It was postulated therefore that there might be alterations of electrolyte concentration in the jejunal tissue of infested rats.

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It was also necessary for subsequent experiments to arrive at a satisfactory means of expressing the rate of absorption or net flux from the intestine. The rate per unit length of intestine would be unsatisfactory because, as has already been stated, the ratio mucosal area per centimetre length was probably not the same for both normal and infested rats. Dry weight of total intestinal tissue, by which is meant mucosal and muscular tissue together, would not be satisfactory because of the hypertrophy of the muscularis externa in the infested animals.

It was therefore decided to express fluxes in terms of the dry weight of the jejunal mucosa. A measurement of this fraction of the intestinal tissue would not only allow fluxes from the jejunum to be referred directly to the functional tissue concerned, but would also provide a quantitative confirmation of the increase already reported (Symons 1957).

In this part, therefore, are included electrolyte, water, whole blood, and fat estimations, as well as the dry weight of the jejunal tissue of normal and infested rats.

## II. METHODS

The rats weighed between 120–200 g. Infestations were produced by the method already described (Symons 1957) and the rats were killed on the tenth day of the infestation for reasons already given (op. cit. p. 375).

### (a) *Dry Weight of Mucosal Tissue per Centimetre Length of Jejunum*

(i) *Dry Weight of Total Jejunal Tissue (mucosa plus muscle layers).*—The jejunum of 18 normal and 18 infested rats, which were fasted and anaesthetized with urethane, were washed out with warm isotonic saline solution. The rats were killed and bled by severing the great vessels in the thoracic cavity. The jejunum were carefully freed from the mesentery and removed to an enamel tray. After a segment of 8 cm had been isolated from below the duodeno-ileal flexure it was opened longitudinally and lightly scraped to remove all mucous and debris. These segments were placed in groups of three in small weighed petri dishes and dried to constant weight by heating for 24 hr at 100°C. Six samples of both normal and infested rats, each containing three segments of 8 cm, were therefore obtained by this method.

(ii) *Dry Weight of Jejunal Smooth Muscle.*—Eighteen rats of each group were treated as above except that the mucosal layer was removed by scraping with the edge of a microscope slide. Histological examination showed that this removed all the mucosa except that in the depths of the crypts.

The dry weight of the mucosa per centimetre length of jejunum could then be calculated for both normal and infested rats from the difference between the mean dry weights of the six samples of total and muscular tissue.

### (b) *Jejunal Tissue Constituents*

(i) *Sodium, Potassium, Chloride, Water, and Fat.*—Except for blood content, which was estimated separately, extractions were made for fat and for the electrolytes sodium, potassium, and chloride from 45 male rats in both the control and infested groups. They were grouped in samples of three, for accuracy and convenience, so that finally there were 15 estimates for comparison. In addition it

was necessary to divide the work into three separate batches spread over about as many weeks. At all times, however, three infested rats were killed alternately with three controls.

The rats which were not fasted were killed by a blow on the head under light ether anaesthesia. They were not bled. The alimentary tract was quickly removed and a segment about 9 cm distal to the pylorus and about 30 cm long was isolated. This length of segment included all that part of the small intestine which was infested by the parasites.

After the external blood had been washed off with distilled water, the segment was laid on a sheet of blotting paper, slit open longitudinally, and the contents lightly scraped off with a scalpel. Most of the parasites were removed by this procedure. The intestine was then blotted to remove any surface fluid and placed in a closed petri dish of known weight together with two similar segments to complete the group of three. After weighing, the samples were then dried to constant weight at about 100°C. It was found that 24 hr drying was sufficient. Before reweighing after drying, the petri dishes were allowed to equilibrate with room temperature over calcium chloride in a desiccator.

The subsequent extractions were based on those described by Lowry and Hastings (1942). The samples were ground with a mortar and pestle, transferred to weighed and stoppered 100-ml Erlenmeyer flasks, redried, and shaken with ether. After standing overnight the ether was drawn off and more added with further shaking. This ether was drawn off after a further 3-4 hr and the sample redried, first in a draught through a fume cupboard and finally at about 100°C. The percentage of fat on a dry weight basis was calculated by difference.

The defatted tissue was stored in test tubes at about -10°C until extracted for electrolytes. About 550 mg of this tissue was weighed into 300-ml Erlenmeyer flasks and redried. 20 ml 0.75N  $\text{HNO}_3$  per 500 mg tissue was added and, to allow for evaporation which had been calculated previously, a further 0.35 ml  $\text{HNO}_3$  added. Extraction was by the open Carius method described by Castor, MacDonald, and Armstrong (1955) without the addition of the reagents for chloride estimation. The extraction was carried out in an autoclave for 2 hr at 15 lb/sq.in. pressure. A watch-glass was placed over the mouth of the flask during this process. After cooling, the mixture was centrifuged and the supernatant was drawn off, quick-frozen in test tubes immersed in dry ice and ethanol, and stored at about -10°C.

For sodium and potassium analyses, two 5-ml aliquots of this supernatant were evaporated in 100-ml Erlenmeyer flasks over a hotplate to remove all nitric acid. The evaporation was repeated once by adding a little distilled water. The residue was finally diluted to 50 ml and the concentration of these two electrolytes then determined using the flame photometric attachment to a Beckman DU spectrophotometer and expressed as m-equiv/kg fresh tissue and m-equiv/100 g of dry fat-free tissue.

Chloride analyses were made in duplicate without prior evaporation of the supernatant by the Volhard method as modified by Lowry and Hastings (1942), and expressed on the same fresh weight and dry fat-free bases as were the other electrolytes.

(ii) *Whole Blood*.—The whole blood content of control and infested tissue was calculated using slight modifications of the acid haematin method of Goodman, Lewis, and Schuck (1951).

Segments of small intestine were prepared by the same method as described above. Eighteen infested rats were used to prepare nine paired samples, but it was necessary to use four animals per sample for the normal rats, i.e. 36 rats were used. As each sample was prepared it was transferred to a cold room at about 4°C. All subsequent operations were carried out in this room with cold glassware and reagents.

The samples were homogenized in 100 ml of distilled water in a Waring Blendor and 3 ml homogenate added, in duplicate, to 3 ml of the phosphotungstic acid reagent of Crooke and Morris (1942) in 15-ml graduated centrifuge tubes. 5 ml ether and then 2 ml distilled water were added. The mixture was thoroughly shaken between each step. After centrifuging for 5 min at 3500 r.p.m. the volume of the ether phase was recorded before it was carefully transferred to stoppered cuvettes and the transmission read at 400  $m\mu$  in a Bausch and Lomb spectrometer against ether as a blank.

Standard curves were prepared separately for the control and infested groups. Rats were bled in the cold from the carotid arteries and jugular veins into a drop of heparin. From this blood 1.25 ml was diluted to 500 ml to give a concentration of 0.25 ml/100 ml. Further dilutions were made from this to give a series from 0.05 ml to 0.25 ml/100 ml in steps of 0.05 ml. These standards were then treated as described above.

All photometry was carried out at room temperature. It was convenient to correct the percentage transmission to that for 5 ml of ether phase, assuming a linear relationship between transmission and concentration. The volume of the ether phase never varied by more than  $\pm 0.3$  ml and usually by less than this so that any error introduced by this practice would be negligible.

This method does not make an absolute measurement of acid haematin but it does make a valid estimation of blood volume which was expressed as ml/kg fresh tissue.

(iii) *Cleaning Glassware for Electrolyte Analyses*.—All test and centrifuge tubes were cleaned with fuming nitric acid and absolute ethanol in a fume cupboard. They were then given several washings in tap water and then distilled water and dried. Other glassware was soaked in equal parts of concentrated nitric acid and water before washing in tap and distilled water. Pipettes were washed by drawing through them successively nitric acid, ethanol, and ether.

### III. RESULTS

#### (a) *Dry Weight of Mucosal Tissue per Centimetre Length of Jejunum*

The dry weight of the mucosal tissue per centimetre of jejunum together with the dry weight of total jejunal tissue per centimetre are set out in Table 1. In infested animals both ratios are increased by 50 per cent. and these differences are highly significant ( $P < 0.001$ ). This increase in the dry weight of total tissue of

the jejunum confirms the earlier finding of Symons (1957). The increase in the weight of the mucosal tissue is a quantitative confirmation of the observation made in the same report. The significance of this to measurements of fluxes from the jejunum is discussed below.

TABLE 1  
DRY WEIGHTS OF TOTAL AND MUCOSAL TISSUE PER CENTI-  
METRE LENGTH OF JEJUNUM OF NORMAL AND INFESTED  
RATS

	Total Tissue (g/cm)	Mucosal Tissue (g/cm)
Normal rats	$0.021 \pm 0.003$	$0.015 \pm 0.003$
Infested rats	$0.033 \pm 0.002$	$0.023 \pm 0.002$

(b) *Jejunal Tissue Constituents*

The water content of the jejunal tissue on a fresh weight basis increased from  $78.1 \pm 0.8$  g/100 g tissue for normal rats to  $80.6 \pm 0.4$  g/100 g tissue for infested rats. Fat content on a dry weight basis decreased from  $16.1 \pm 3.3$  to  $12.2 \pm 1.9$  g/100 g tissue for normal and infested rats respectively. Both differences are highly significant ( $P < 0.001$ ).

TABLE 2  
ELECTROLYTE CONSTITUENTS OF JEJUNAL TISSUE IN NORMAL AND INFESTED RATS

	Sodium	Potassium	Chloride
m-equiv/kg tissue on wet weight basis			
Normal rats	$43.8 \pm 3.4$	$96.3 \pm 5.7$	$39.1 \pm 1.7$
Infested rats	$48.6 \pm 2.8$	$95.4 \pm 7.0$	$45.7 \pm 2.0$
	$\left. \begin{array}{l} 43.8 \pm 3.4 \\ 48.6 \pm 2.8 \end{array} \right\} P < 0.001$	$\left. \begin{array}{l} 96.3 \pm 5.7 \\ 95.4 \pm 7.0 \end{array} \right\} \text{n.s.}$	$\left. \begin{array}{l} 39.1 \pm 1.7 \\ 45.7 \pm 2.0 \end{array} \right\} P < 0.001$
m-equiv/100 g fat-free tissue on dry weight basis			
Normal rats	$23.8 \pm 2.2$	$52.4 \pm 2.6$	$21.2 \pm 1.0$
Infested rats	$28.6 \pm 2.0$	$56.1 \pm 3.8$	$26.9 \pm 1.6$
	$\left. \begin{array}{l} 23.8 \pm 2.2 \\ 28.6 \pm 2.0 \end{array} \right\} P < 0.001$	$\left. \begin{array}{l} 52.4 \pm 2.6 \\ 56.1 \pm 3.8 \end{array} \right\} P < 0.01$	$\left. \begin{array}{l} 21.2 \pm 1.0 \\ 26.9 \pm 1.6 \end{array} \right\} P < 0.001$

In Table 2 are shown the sodium, potassium, and chloride concentrations in the jejunum of both normal and infested groups expressed as m-equiv/kg fresh weight and as m-equiv/100 g fat-free solids. The standard deviations and the results of *t*-tests of the differences between normal and infested rats are included.

These values for normal tissue are in close agreement with those of Grollman (1954) who found 43.5 m-equiv. Na<sup>+</sup>/kg blood-free, fat-free wet tissue and 103.1 m-equiv. K<sup>+</sup>/kg "gut" tissue of dogs. If these are corrected to fresh tissue on the



basis of the concentrations of fat and blood found in the present work these values become *c.* 42 m-equiv. Na<sup>+</sup>/kg and 99 m-equiv. K<sup>+</sup>/kg respectively. Manery and Bale (1941) reported 40·7 m-equiv. Cl<sup>-</sup>/kg and 47·7 m-equiv. Na<sup>+</sup>/kg fresh weight in rabbit small intestine.

It can be seen from Table 2 that there was an increase in the concentration of the sodium and chloride ions when measured on both a fresh weight and dry weight, fat-free basis. There is no difference in the potassium content when measured on a fresh weight basis but it is increased significantly when expressed as concentration per 100 g fat-free tissue. This latter result is probably due to the fact that the increased water content, which histologically can be seen to be due largely to oedema of the mucosa and is therefore extracellular fluid presumably containing little potassium, would tend to mask any increase of that element when measured in fresh tissue.

The concentrations of whole blood in the jejunal tissue increased from  $13\cdot2 \pm 2\cdot7$  ml/kg tissue for normal rats to  $22\cdot5 \pm 2\cdot0$  ml/kg tissue for infested rats on a fresh weight basis. The difference, which is again highly significant ( $P < 0\cdot001$ ), shows that there is nearly a twofold increase in the concentration of blood in infested tissue. The obvious congestion of the intestine certainly suggested that this might be so.

#### IV. DISCUSSION

The 50 per cent. increases of the dry weights of jejunal tissue and of mucosal tissue alone from the same organ is in agreement with that reported earlier for the entire small intestine (Symons 1957). The ratio mucosal dry weight per centimetre length of jejunum also provides a means of expressing fluxes or absorption from this section of the gut provided the length is known. It must be emphasized that this ratio refers to the jejunum alone. Fisher and Parsons (1950) have shown that the ratio of surface area of mucosa to length of intestine in the rat increases with the distance proximal to the ileo-caecal valve. These authors described a method of measuring mucosal area but it was not possible to apply it to these infested rats because of the irregular shape of the villi.

It has already been pointed out (see Section I) that neither total dry weight of the total small intestine nor its length would provide a basis of comparison for absorption rates between normal and infested rats. These results bear this out. The serosal area can be measured quite simply, but this would provide only an artificial measure of absorptive rate and would depend upon a similar relationship between serosal and mucosal areas in both groups of rats. It might be objected that mucosal dry weight is not a precise measure of functional tissue in an infested animal if there is damage to some epithelial cells but not to others. It is, however, difficult to envisage any other method which would more accurately relate function to the tissue concerned.

The consequence of the difference in this ratio of mucosal dry weight to length between normal and infested rats is of some importance to absorption studies. Absorption expressed per unit of dry weight would provide a comparison of function per unit of mucosa. On the other hand, the rate of absorption per centimetre length

would compare what might be called the gross rate of absorption from identical lengths of intestine bearing different areas of mucosa. In fact these differences were subsequently found to be important (Symons 1960).

The increase in the water content of the jejunum confirms the earlier report of a similar increase in the small intestine as a whole. The fall in fat content of the same organ has also been found to be true of the carcass as a whole (Symons 1959).

It is highly unlikely that the increases in electrolyte concentrations are due to the inclusion of parasites in the dried and ground tissue. The great majority of these were removed. The few that remained would constitute a very small fraction of the total weight of tissue for they are hair-like and 4–5 mm in length. Calculations were made to ascertain whether the different proportions of water, blood, and solids in the infested tissue could possibly account for these electrolyte changes. It was assumed that in these calculations the additional water was extracellular fluid containing little potassium, and that whole blood contained the normal proportions of the electrolytes. It has been reported by Symons (1959) that the serum electrolytes are not affected by the infestation. When these estimations were made, it was found that unless the actual concentrations of electrolytes in the tissue were increased, the altered proportions of water, blood, and solids would have produced an increase of only 1.2 m-equiv.  $\text{Na}^+$ /kg and losses of 3.1 m-equiv.  $\text{K}^+$ /kg and 0.3 m-equiv.  $\text{Cl}^-$ /kg of fresh weight.

There is no reasonable doubt that the increases reported are real but it is difficult to assess their physiological significance. Certainly it can be stated confidently that there is no loss of potassium which might cause a loss of motor activity of the gut. The concentrations of electrolytes shown in Table 2 refer to the mucosal and muscular layers together. No attempt has been made to measure them separately nor to divide them compartmentally between the extra- and intracellular fractions. An assessment of the physiological significance of the changes in the infested rats would depend upon such examinations.

It is interesting, however, to compare these results with those of Widdowson and McCance (1956) and Huth and Elkington (1959) who measured electrolyte levels in various tissues in starved or otherwise undernourished rats. The former authors found that there was a gain in the chloride and sodium content of the liver in both starved and chronically undernourished rats. The change in the potassium level was indefinite. The total body chloride and sodium levels on a fat-free wet basis were found by the latter two investigators to have increased in starved rats, while the potassium level was significantly decreased. Both groups assumed that there was an increase of extracellular water if this can be defined by the chloride space. Huth and Elkington, however, suggested that the additional chloride could be due alternatively to a sequestration of that electrolyte. These two also reported that there was a fall of potassium relative to total water in the carcasses of starved rats. These ratios for the jejunum of normal and infested rats in the examinations described here were 0.124 m-equiv/ml and 0.118 m-equiv/ml respectively, which are not significantly different. It has been shown by Symons (1959) that under-nutrition, if not starvation, in severely infested rats is a major factor in the symptomatology of infestation by *N. muris*. The loss of fat from the jejunum supports

this observation. There can be a marked loss of appetite. It is possible, therefore, that the electrolyte changes in the jejunum reported here are a reflection of the same changes found in other tissues both by Widdowson and McCance and by Huth and Elkington and that they are due to undernourishment.

#### V. ACKNOWLEDGMENTS

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# PATHOLOGY OF INFESTATION OF THE RAT WITH *NIPPOSTRONGYLUS MURIS* (YOKOGAWA)

## III. JEJUNAL FLUXES IN VIVO OF WATER, SODIUM, AND CHLORIDE

By L. E. A. SYMONS\*

[Manuscript received November 6, 1959]

### Summary

The net fluxes of water, sodium, and chloride were measured *in vivo* by perfusion of the jejunum. There was a net absorption of these three substances from isotonic saline solutions in normal rats, but a net influx to the lumen in each instance in rats infested with the nematode *Nippostrongylus muris*. The unidirectional fluxes of sodium and the net fluxes during perfusion with hypo- and hypertonic saline solutions indicated that this was fundamentally due to a derangement of efflux while influx was unaffected. The gross effect, however, was also due to an increase of influx because of the greater weight of mucosal tissue per centimetre of jejunum in the infested animal. The unidirectional fluxes of water did not support these conclusions unequivocally. The fluid which accumulates in the infested small intestine can be explained by these results.

### I. INTRODUCTION

It has been shown in Part I of this series (Symons 1957) that there was a considerable increase in the fluid in the lumen of the small intestine of rats infested by the nematode *Nippostrongylus muris* (Yokogawa, 1920). This suggested that there was either a hypersecretion into the gut, a failure of absorption, or a combination of both these factors.

Baker (1955) has suggested that one cause of the anaemia of mice associated with the infestation with *Nematospiroides dubius* may be a deficient absorption as a result of the atony, dilatation, and inflammation which occur in the small intestine. Lesions of this nature occur in nippostrongylosis of rats (Symons 1957).

Indirect measurements by means of balance studies had indicated that intestinal infestations in various hosts may affect absorption. Stewart (1933), Shearer and Stewart (1933), and Franklin, Gordon, and Macgregor (1946) have shown a decrease of crude protein digestion and net mineral absorption by sheep infested with a number of species of nematode. Rogers (1942) found impairment of mineral absorption by rats infested with *Trichinella spiralis* in the intestinal phases. Andrews, Kauffman, and Davis (1944) could not confirm that absorption was affected in sheep infested with *Trichostrongylus colubriformis*.

Direct measurement of movements across the jejunal mucosa, the site of the infestation, was therefore considered to be an important part of the investigation of the pathology of nippostrongylosis. The net fluxes of water, sodium, and chloride, as well as the unidirectional fluxes of the first two substances were measured *in vivo* by a perfusion technique.

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## II. METHODS

(a) *Net Jejunal Fluxes*

(i) *Animals*.—Albino rats from a colony inbred for many generations were used in all experiments. They were infested when they weighed about 120–150 g by the subcutaneous injection of about 3500 larvae of *N. muris*. Most experiments were carried out on the tenth day of the infestation for the reasons given in Part I (Symons 1957). Some perfusions were performed on the ninth or eleventh days as it was not always possible to perfuse sufficient rats from one batch on one day. There was no reason to believe that this introduced any variation into the results. Non-infested controls were perfused in every experiment.

All rats were fasted overnight and anaesthetized with urethane given intraperitoneally at approximately 0.1 mg/100 g body weight. Not only was this anaesthetic easy to administer and long acting, but Alvarez and Hosoi (1929) have shown that it had a negligible effect upon the motility of the gut.

The severity of the infestation was judged subjectively from the appearance of the rat and its jejunum.

(ii) *Perfusion*.—A modified version of the *in vivo* perfusion technique of Curran and Solomon (1957) was used to measure the net fluxes of water and of the sodium and chloride ions across the jejunum of normal and infested rats from various solutions of sodium chloride.

Haemoglobin could not be used as a marker for the volume of solution perfused as it was possible that blood is lost into the lumen of the intestine of infested rats. A high quality burette from which the tap had been removed was supported in a "Perspex" tank. At the end of the burette was attached a No. 25 gauge hypodermic needle which pierced a rubber stopper in the side of the tank. To the outer end of this needle was attached a cannula made of "Polythene" tubing with an original internal diameter of 0.5 mm. This was reduced by stretching. This cannula ended in another No. 25 or 26 gauge hypodermic needle from which the base had been removed. This needle was inserted in the loop of intestine to be tested.

Water in the tank was mixed and heated to 40°C by a constant-temperature heater, which was also used to circulate water through a small copper operating table upon which the rat was laid during the experiment. Also in the tank was a glass vessel to wash out the intestinal loop with the solution to be perfused.

Into the distal end of the loop being tested was inserted a "Polythene" cannula with an internal diameter of 2 mm. This drained into a high quality 10 ml graduated centrifuge tube. The centrifuge tubes used were all calibrated against the volume delivered by the burette between the 14 and 19 ml marks. The volumes perfused were therefore measured directly from the burette. Perfusion was always commenced from the 14.0 ml mark and proceeded under hydrostatic pressure at the rate of about 0.2–0.4 ml/min. This pressure varied from approximately 27 cm of water at the commencement, to 21 cm at the end of each perfusing period of 15 min.

After the abdomen of the rat was clipped the jejunum was exteriorized through a laparotomy. A ligature was tied just distal to the duodeno-ileal flexure and a transection of the intestine made at the suitable distance below this. The aim was

to include the section of maximum infestation in this loop. After the loop had been washed out with the appropriate solution, the distal cannula was tied into the transected end and the proximal cannula inserted through the wall of the intestine below the initial ligature. It was not necessary to ligate this cannula as the intestine did not leak.

Kinking of the segment was avoided by laying it on the abdominal wall rather than by returning it to the cavity. A thermometer was inserted beneath it after which it was covered with a piece of tissue moistened with the perfusing solution. Over this was placed a thin "Polythene" sheet. The preparation was kept warm by placing an electric blanket over all.

About 10–15 min of perfusion was allowed as an equilibrium period during which time the temperature of the loop was raised and kept at  $38^{\circ} \pm 0.5^{\circ}\text{C}$ . After this interval the loop was perfused for three periods of 15 min.

At the completion of the perfusion the rat was killed by inserting scissors into the thoracic cavity to sever the great vessels. This bled the segment which was carefully freed from its mesentery and its length measured to the nearest 0.5 cm. The segments were usually about 7 cm long. Care was taken to standardize this procedure. Peristalsis was still present at the end of the perfusion.

Duplicate aliquots of the perfusate were diluted 1 in 100 and 1 in 25 for sodium and chloride analyses respectively. The diluted samples together with samples of the perfused solution were then quick-frozen in dry ice and ethanol and stored. The sodium concentration was estimated by the flame photometric attachment to a Beckman DU spectrophotometer and the chloride by the method of Schales and Schales (1941) without protein precipitation.

The calculations of the net fluxes were essentially those of Visscher *et al.* (1944) except that they were expressed as millilitres of water or milliequivalents of sodium and chloride per centimetre length of jejunum and per gram of mucosal tissue per hour. The ratio of length of jejunum to dry weight of mucosa for normal and infested rats had been calculated previously (Symons 1960a). The rate of net flux was calculated for each 15-min period and the mean of the three taken as the rate for each rat. Six normal and six infested rats were used in each experiment.

#### (b) *Unidirectional Fluxes*

(i) *Sodium*.—The procedure was identical with that described above except that an approximately isotonic saline solution containing  $^{24}\text{Na}$  prepared by the Australian Atomic Energy Commission was perfused. The solution when freshly prepared contained about  $6\ \mu\text{c/l}$ . These perfusions were carried out in two batches with freshly irradiated sodium in each and with an infested rat alternating with a normal control. Six rats of each group were again used. Net sodium and water fluxes were calculated as before.

Duplicate 1-ml aliquots of the perfusate were placed in aluminium planchettes to which a drop of detergent had been added. These were carefully dried on a hotplate and the number of counts made in 10 min was recorded. A Geiger-Müller counter with a halogen-quenched end-window tube was used for this procedure. Corrections were made for background and decay, but self-absorption was ignored

as it was estimated to make a negligible difference to the subsequent calculations of efflux which were made according to the formula of Visscher *et al.* (1944). Some of these calculations were checked using the formulae of Curran and Solomon (1957) but were found to be identical except in the special instance where the net water flux was zero. In this instance the Curran and Solomon formulae would also give a zero sodium efflux which would not necessarily be true. Influxes were estimated by the difference between net fluxes and effluxes.

(ii) *Water*.—The procedure was again identical with that used before except that D<sub>2</sub>O (99.7 per cent. pure) was added to isotonic saline to give a concentration of D<sub>2</sub>O of about 3 per cent. (w/w). During each half of the experiment a freshly made solution was kept in a stoppered measuring cylinder from which the burette of the perfusing apparatus was replenished before each perfusion. An aliquot was also taken for analysis after the perfusion of each rat. The experiment was again divided into two daily periods in which six rats were perfused in each and an infested rat alternated with a normal rat.

To reduce exchange of D<sub>2</sub>O with the atmosphere a rubber cap with a hole just large enough to take the distal cannula was fitted over the top of the centrifuge tube in which the perfusate was collected. This tube was closed with a rubber stopper immediately after perfusion. Shortly afterwards duplicate samples were quickly withdrawn for sodium and chloride analyses. The remainder was transferred to small vaccine bottles with special close-fitting rubber caps and stored at about 4°C. Analysis of D<sub>2</sub>O concentrations in these samples was carried out by the Coal Research Section, C.S.I.R.O., using a Perkin-Elmer infra-red spectrometer, No. 112. The percentage D<sub>2</sub>O (w/w) was estimated by measuring the intensity of the HOD band ( $\text{H}_2\text{O} + \text{D}_2\text{O} \rightleftharpoons 2 \text{HOD}$ ) at  $3.98 \mu$  with a slit width of 0.01 mm (Gaunt 1956). The cell used had calcium fluoride windows with a fixed light path of about 0.02 mm.

Efflux was calculated by the formula of Visscher *et al.* (1944) and influx by difference from the net flux.

All tests of significance were made using the *t*-test and standard deviations were calculated.

### III. RESULTS

#### (a) *Net Jejunal Fluxes*

In Table 1 are shown the net fluxes of water, sodium, and chloride for six normal and six infested rats perfused with solutions containing 72, 142, and 280 m-equiv. NaCl/l. The statistical differences refer to comparisons of the hypo- and hypertonic perfusions with that of 142 m-equiv. NaCl/l. All results are expressed in terms of net flux/hr/g of dry mucosal tissue. For reasons given by Symons (1960a) this provides a measure of flux per unit of functional tissue. The plus sign indicates net absorption from the lumen and the minus sign a net influx into the lumen.

There was a net absorption of water and of both ions from the jejunum of normal rats perfused with a solution containing 142 m-equiv. NaCl/l. The infestation, on the other hand, reversed the direction of movement so that there were net influxes of water and electrolytes. These differences between normal and infested rats are highly significant.



It has been shown by Visscher *et al.* (1944) and confirmed by Curran and Solomon (1957) that water from a hypotonic saline solution is very rapidly absorbed in response to the osmotic gradient between the lumen and plasma. Conversely water was found to enter the lumen during absorption from a strongly hypertonic solution. In these experiments this was again found to be true for normal rats, when the rates were highly significantly different from those for perfusion with an approximately isotonic saline solution. The absorption of the sodium ion was not

TABLE 1  
NET FLUXES PER HOUR PER GRAM DRY MUCOSAL TISSUE FROM JEJUNAL LOOPS OF NORMAL AND INFESTED RATS PERFUSED IN VIVO WITH SALINE SOLUTIONS  
+ = net absorption from the lumen; - = net influx to the lumen. Statistical differences refer to comparisons of isotonic with hypo- and hypertonic saline perfusions

Saline Concn. (m-equiv/l)	Water (ml)	Sodium (m-equiv.)	Chloride (m-equiv.)
Normal rats			
72	$+21.03 \pm 4.53$	$+0.25 \pm 0.36$	$+0.77 \pm 0.39$
142	$+7.58 \pm 2.87$	$+0.63 \pm 0.62$	$+1.18 \pm 0.61$
280	$-8.11 \pm 5.44$	$+0.70 \pm 1.56$	$+3.58 \pm 1.90$
	$\left. \begin{array}{l} P < 0.001 \\ P < 0.001 \end{array} \right\}$	$\left. \begin{array}{l} \text{n.s.} \\ \text{n.s.} \end{array} \right\}$	$\left. \begin{array}{l} \text{n.s.} \\ P < 0.05 \end{array} \right\}$
Infested rats			
72	$-7.39 \pm 2.95$	$-2.10 \pm 0.28$	$-1.57 \pm 0.25$
142	$-5.50 \pm 3.82$	$-1.10 \pm 0.41$	$-0.51 \pm 0.68$
280	$-12.70 \pm 3.20$	$-0.95 \pm 1.45$	$+0.95 \pm 0.69$
	$\left. \begin{array}{l} \text{n.s.} \\ P < 0.01 \end{array} \right\}$	$\left. \begin{array}{l} P < 0.001 \\ \text{n.s.} \end{array} \right\}$	$\left. \begin{array}{l} P < 0.01 \\ P < 0.01 \end{array} \right\}$

significantly changed by perfusion with hypo- and hypertonic solutions. This is again in conformity with the results of Curran and Solomon's experiments when they found a poor response by sodium to osmotic gradients. The small changes that were recorded were, however, in the directions that could be predicted. The changes in the net fluxes of chloride were also in the predicted directions, but only the increase in response to hypertonic perfusion reached statistical significance ( $P < 0.05$ ).

There was no significant change in the rate of net water influx during perfusion of infested jejunum with hypotonic solution but the increase during hypertonic perfusion was in the predicted direction and was statistically significant ( $P < 0.01$ ). This strongly suggests that efflux was affected rather than influx. The rates of net electrolyte fluxes were not quite so unequivocal, although the significant increase in net influx of sodium during hypotonic perfusion did support the evidence from the net water fluxes. The net chloride influxes were also increased during hypotonic



perfusion but they became net effluxes during hypertonic perfusion, which did not indicate any marked inability to absorb. The response in this instance was not as great as that recorded for normal rats.

(b) *Unidirectional Fluxes*

(i) *Sodium*.—The results of experiments in which jejunal loops of normal and infested rats were perfused with solutions containing  $^{24}\text{Na}$ -labelled  $\text{NaCl}$  at a concentration of about 140 m-equiv/l are shown in Table 2. These are expressed as fluxes/hr/cm length of intestine as well as per gram of dry mucosal tissue. The

TABLE 2

SODIUM FLUXES FROM JEJUNAL LOOP OF NORMAL AND INFESTED RATS PERFUSED WITH SODIUM CHLORIDE SOLUTION LABELLED WITH RADIOSODIUM  
Sodium chloride concentration 140 m-equiv/l

	Net Flux*	Influx	Efflux
m-equiv. sodium/hr/g dry mucosal tissue			
Normal	$+1.7 \pm 0.8$	$3.3 \pm 1.4$	$5.0 \pm 1.7$
Infested	$-1.4 \pm 0.7$	$3.3 \pm 1.0$	$1.9 \pm 0.9$
	$P < 0.001$	n.s.	$P < 0.01$
m-equiv. sodium/hr/cm jejunum ( $\times 10^2$ )			
Normal	$+2.56 \pm 0.67$	$5.01 \pm 0.58$	$7.57 \pm 1.20$
Infested	$-3.15 \pm 1.52$	$7.65 \pm 1.74$	$4.51 \pm 1.45$
	$P < 0.001$	$P < 0.01$	$P < 0.01$

\* + = net efflux; - = net influx.

latter is a measure of the rate of flux per unit functional tissue, while the former allows for the fact that there is actually more mucosal tissue per centimetre of jejunum in the infested rat.

It can be seen that per unit weight of tissue the rate of influx was the same in both normal and infested animals. On the other hand, the rate of efflux in the infested rat was reduced to nearly a third of that in the normal rat. This confirmed the indications reported in the previous section that a derangement of efflux accounted for the net influx found for isotonic perfusion. Once again the net fluxes were in the same directions as previously found although they differed in magnitude. The net efflux in normal rats especially was considerably greater.

The sodium fluxes per centimetre of jejunum are important as they are measurements of what actually happens in the jejunum as a whole. The influx of sodium was then greater in the infested animal. For the same reason the total efflux per centimetre was not so markedly reduced by the infestation when only the effluxes per unit of mucosal tissue were considered.

(ii) *Water*.—The rates of net flux, influx, and efflux of water in normal and infested rats are set out in Table 3 where they are again expressed in terms of dry weight of mucosal tissue and centimetre length of jejunum.

It was not possible to measure the unidirectional movement of water concurrently with sodium because the radiosodium and the infra-red spectrometer for deuterium analysis were not available at the same time.

The results do not show the same clearly defined derangement of efflux from the lumen found with sodium. The reduction of water efflux/hr/g dry mucosal tissue was not statistically significant and suggested that the infestation reduced it by

TABLE 3  
WATER FLUXES FROM JEJUNAL LOOPS OF NORMAL AND INFESTED RATS PERFUSED WITH SODIUM CHLORIDE SOLUTION CONTAINING D<sub>2</sub>O  
Sodium chloride concentration 140 m-equiv/l

	Net Flux*	Influx	Efflux
ml water/hr/g dry mucosal tissue			
Normal	$+10.5 \pm 4.3$	$33.2 \pm 6.6$	$43.7 \pm 7.0$
Infested	$-4.3 \pm 1.8$	$37.7 \pm 11.1$	$33.4 \pm 10.4$
	$P < 0.001$	n.s.	n.s.
ml water/hr/cm jejunum ( $\times 10$ )			
Normal	$+1.57 \pm 0.64$	$4.99 \pm 0.99$	$6.56 \pm 1.04$
Infested	$-0.99 \pm 0.40$	$8.67 \pm 2.56$	$7.68 \pm 2.39$
	$P < 0.001$	$P < 0.01$	n.s.

\* + = net efflux; - = net influx.

about one-third rather than to one-third. The influx was again not affected. It is important to note, however, that the net water fluxes were again in opposite directions and highly significantly different.

The fluxes of water expressed per centimetre of jejunum again indicated the gross effect due to the greater weight of mucosal tissue per unit length of infested intestine, for the influx then became significantly larger ( $P < 0.01$ ). The possible reasons for this lack of conformity between the unidirectional sodium and water fluxes are discussed below.

#### IV. DISCUSSION

Measurement of the fluxes of water, sodium, and chloride provided strong evidence to account for the fluid which accumulates in the jejunum of the rat infested with *N. muris*. The net fluxes were unequivocally in opposite directions so that there was a net movement of water and of the sodium and chloride ions into the lumen of the infested animal.

Under the conditions of the experiment the jejunum of the normal rat responded to the presence of hypo- and hypertonic solutions in the same manner as did the rats similarly perfused by Curran and Solomon (1957), and the dogs of Visscher *et al.* (1954) in which test solutions were placed in isolated intestinal loops.

In the infested rat the evidence that the net influx during isotonic perfusions was fundamentally due to a derangement of efflux while influx remained constant depended largely upon the unidirectional fluxes of the sodium ion. It was supported by the responses of the jejunum to hypo- and hypertonic solutions. The net water influx was not changed during perfusion of the infested intestine with a solution containing 72 m-equiv. NaCl/l, but it did respond during perfusion with a solution of 280 m-equiv. NaCl/l by increasing significantly the rate of net influx. These differential responses of net water fluxes to osmotic gradients conflict with the unidirectional water fluxes which did not confirm the clearly defined derangement of efflux shown by the radiosodium experiment.

This difference between the two unidirectional flux experiments was rather surprising as Curran and Solomon (1957) had reported a strong correlation between net sodium and water fluxes and had published experimental evidence in confirmation of it. The difference might be explained by the fact that while the rats in the sodium experiment were all heavily to very heavily infested, those in the deuterium experiment were judged to be only moderately to heavily infested. On the other hand, an appreciable loss of deuterium by exchange with the atmosphere would mask any difference between the normal and infested perfusates. However, even without a specially contrived dry box it was calculated that this exchange would be negligible. The collecting centrifuge tube was covered with a rubber cap and the distal cannula was inserted as far down into the tube as possible.

If the results of this experiment are accepted at their face value, the net water influx into the lumen during infestation is fundamentally due to a combination of a small increase of influx and a small decrease of efflux per gram of dry mucosal tissue. The gross effect is due mainly to a large increase of influx because of the greater weight of mucosal tissue per centimetre of jejunum. Furthermore, if these conclusions can be accepted, sodium movement is not a reliable guide to water movement in infested animals, but reasons for doubting these results have been given.

It should be added here that subsequent experiments with glucose (Symons 1960*b*) supported the evidence that absorption from the jejunum is affected by the infestation.

The assumption of Curran and Solomon (1957) that influx represents a constant diffusion into the lumen from a source of constant concentration on the other side of the membrane can be used to explain the accumulation of fluid in the jejunum even in the fasting state, provided that the efflux is impaired. In Table 2 it can be seen that the efflux of sodium in the infested rat is less than the influx whether measured per unit of mucosal tissue or per centimetre of intestine. Similarly, as can be seen in Table 3 the efflux of water tended to be smaller than the influx.

Finally, these results indicate that nematodes can markedly affect the function of the intestine at the site of the infestation.

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# PATHOLOGY OF INFESTATION OF THE RAT WITH *NIPPOSTRONGYLUS MURIS* (YOKOGAWA)

## IV. THE ABSORPTION OF GLUCOSE AND HISTIDINE

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### *Summary*

The rate of absorption of D-glucose and L-histidine from the entire small intestine of the rat when measured by an intubation technique was not affected by infestation with the nematode *Nippostrongylus muris*. On the other hand, absorption of D-glucose from the infested jejunum when measured *in vivo* by a perfusion technique was severely reduced. The rate of gastric emptying was not affected by the infestation. There was a direct relationship between gastric emptying and the rate of absorption of glucose.

## I. INTRODUCTION

References to indirect methods of measuring absorption from animals infested with intestinal nematodes have been given in Part III of this series (Symons 1960*b*). Measurements of fluxes across the jejunum of rats infested with *Nippostrongylus muris* (Yokogawa, 1920) reported in that paper showed clearly that there is a reduced ability to absorb sodium and chloride from the infested section of the intestine. Curran and Solomon (1957) have shown a relationship between sodium and water movement, but it could not be assumed that absorption of other substances was linked to similar mechanisms, or that derangement of one was necessarily true of another. Furthermore, the earlier experiments did not measure absorption from the small intestine as a whole.

Glucose is an end-product of carbohydrate digestion and histidine might be taken as one of the end-products of protein digestion. A knowledge of the absorption of these substances as part of the chain in the utilization of the products of digestion was therefore considered to be an important aspect of this investigation.

Glucose and histidine were used to determine directly whether absorption from the whole small intestine was affected by the infestation, while glucose alone was used to estimate the rate of absorption from the jejunum. The rate of gastric emptying and its correlation with the rate of absorption from the small intestine were also estimated.

## II. METHODS

### (a) *Absorption from the Entire Small Intestine*

Infestations were produced as described by Symons (1957). The rats used weighed between 120–190 g. They were fasted overnight.

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(i) *Glucose Absorption*.—The rats were fed 2 ml of a warm 1M solution of D-glucose by stomach tube under light ether anaesthesia. They were returned to their cage for 40 min and were then killed by bleeding under anaesthesia. The gastro-intestinal tract was removed after ligaturing the oesophagus, pylorus, and ileo-caecal junction. The isolated stomach and small intestine were opened separately into distilled water in 250-ml measuring cylinders which were shaken to free all glucose. The organs were then removed from the cylinders which were filled to 250 ml.

The protein was precipitated from 1-ml duplicate samples of the diluted contents with 2 ml 0.3N  $\text{Ba}(\text{OH})_2$  and 2 ml 5 per cent.  $\text{ZnSO}_4$ . After filtering, the glucose concentration was estimated by the Nelson-Somogyi method (Nelson 1944). The weight of glucose fed was estimated by diluting the 2 ml delivered by the syringe and stomach tube to 1 litre. Aliquots were then treated as before.

The large bowel was discarded as it had previously been determined that no glucose entered this organ in 40 min in either normal or infested rats. The residual glucose in the stomach and small intestine after fasting was found to be negligible which is in agreement with the results of Heller (1954).

(ii) *Histidine Absorption*.—Unanaesthetized rats were fed a warm solution containing 150–180 mg of L-histidine monohydrochloride using the gagging device of Gillespie and Lucas (1957). Subsequent treatment was the same as for glucose absorption except that gastro-intestinal contents were diluted to either 500 or 1000 ml. Residual histidine after fasting was found to be significant and was taken into account in the calculations of the weight absorbed. The large bowel was discarded for the same reason as for glucose.

Histidine was estimated from 5-ml aliquots of the diluted and filtered contents by the method of Macpherson (1946), as modified by Birt and Hird (1956).

#### (b) *Absorption of Glucose in vivo from the Jejunum*

The method used was identical with the perfusion technique described by Symons (1960b) for the measurement of water and electrolyte fluxes. A 0.25M solution of D-glucose in Krebs-Henseleit bicarbonate buffer (Umbreit, Burris, and Stauffer 1949) was perfused for three periods of 15 min in each rat. The concentration of glucose in the perfusing fluid and the perfusate was estimated in duplicate by the Nelson-Somogyi method (Nelson 1944) after dilution to 1 in 50 or 1 in 100.

The possible presence of "endogenous glucose" which might have entered the lumen of the loop during perfusion was tested by perfusing as before with the bicarbonate buffer alone, in both normal and infested rats.

The rate of absorption was expressed as m-moles/hr/g of dry mucosal tissue and as  $\mu$ moles/hr/cm of intestinal loop.

### III. RESULTS

#### (a) *Absorption from the Entire Small Intestine*

(i) *Glucose Absorption*.—The results, expressed as the weight of glucose absorbed in 40 min by 11 normal and 10 infested rats, together with the body weights

and worm counts are set out in Table 1. There was no significant difference between the normal and infested rats which absorbed  $181 \pm 42$  mg and  $200 \pm 20$  mg glucose respectively.

Absorption was expressed in this manner rather than by the more familiar coefficient of absorption of Cori (1925), which relates the weight absorbed per hour to 100 g body weight, as Fenton (1945) could not confirm that absorption was constant with time and linearly correlated with body weight. Rats of similar body weight were used to avoid any possible confusion by this factor and, in fact, the means of the body weights of both the normal and infested rats were 161 g. The worm burdens for all but one rat were considered to be heavy.

TABLE 1  
ABSORPTION OF GLUCOSE IN NORMAL AND INFESTED RATS

Rat No.	Normal Rats		Infested Rats		
	Body Weight (g)	Absorption (mg/40 min)	Body Weight (g)	Absorption (mg/40 min)	Worm Counts
1	159	140	176	209	1500
2	156	209	159	206	2900
3	157	166	177	206	1400
4	145	223	140	154	1600
5	163	135	140	185	2900
6	144	207	155	214	1700
7	180	154	165	221	1200
8	152	141	157	214	2200
9	164	183	182	181	1000
10	180	261	158	210	1700
11	173	171	—	—	—
Mean	$161 \pm 12$	$181 \pm 42$	$161 \pm 14$	$200 \pm 20$	

(ii) *Histidine Absorption*.—In Table 2 is set out the residual histidine in the stomach and small intestine of 5 normal and 5 infested rats after fasting overnight. This was regarded as negligible for the stomachs of both groups. On the other hand, approximately 2 and 3 mg, respectively, were found to remain in the small intestines of the normal and infested rats and were deducted from the appropriate recoveries from the experimental animals.

The weights of histidine monohydrochloride absorbed in 40 min by 9 normal and 11 infested rats are shown in Table 3 from which it can be seen that  $120 \pm 10$  mg and  $114 \pm 21$  mg were absorbed by the normal and infested rats respectively. These values are not significantly different.

No explanation can be offered for the fact that the amounts absorbed by two of the infested rats were markedly lower than the mean. One of these (70 mg per 40 min) was very heavily infested (2000 worms) while the other (88 mg per 40 min)

was moderately infested (900 worms). On the other hand, rats almost as heavily infested as the first of these two absorbed histidine as readily as the non-infested or other moderately infested animals. As can be seen from Table 3 the infestations were again mostly heavy.

(iii) *Rates of Gastric Emptying and of Absorption.*—As the weights of both glucose and histidine leaving the stomach in 40 min could be measured, it was possible to calculate the percentage of the weight fed which left the stomach and its possible relationship with the subsequent rate of absorption. In Table 4 is shown the percentage of the weight fed which entered the small intestine for both glucose

TABLE 2  
RESIDUAL HISTIDINE IN THE STOMACH AND SMALL INTESTINE OF FASTED NORMAL AND INFESTED RATS

Rat No	Normal Rats		Infested Rats	
	Stomach Residue (mg)	Small Intestine Residue (mg)	Stomach Residue (mg)	Small Intestine Residue (mg)
1	0.20	2.44	0.28	3.36
2	0	2.16	0.12	2.88
3	0.08	1.28	0.08	2.52
4	0.20	2.00	0.20	2.40
5	0	1.84	0.08	2.40
Mean	0.1	1.94	0.15	2.71

and histidine in normal and infested rats. The rates were  $62.8 \pm 12.2$  per cent. and  $71.7 \pm 11.2$  per cent. respectively for glucose and  $86.9 \pm 3.7$  per cent. and  $89.8 \pm 7.5$  per cent. for histidine.

As there was no significant difference between the gastric emptying rates of the normal and infested rats for either of the substances tested, the results of the two groups were combined in both instances to calculate the relationship between the gastric emptying rate and absorption. There was a highly significant positive correlation between the rate of entry into the small intestine and absorption of glucose ( $P < 0.001$ ), but not of histidine. This correlation between gastric emptying and glucose absorption agrees with the finding of Birchall, Fenton, and Pierce (1946) and Reynell and Spray (1956).

(b) *Glucose Absorption in vivo from the Jejunum*

The original experiment used six rats in both the infested and control groups, but as the variation between rats, in fact even the variation between perfusion



periods in one rat, was often large, it was thought that a fault in the technique of glucose estimation may have been responsible. Fullerton and Parsons (1956), who also perfused small intestines *in vivo*, commented on the marked variation between rats. Another six rats in each group were used and a small modification made in the technique. An analysis of variance which was made upon these two collections of data revealed that the variation between the two experiments was in fact smaller than the variation between rats. Furthermore, the variation between infested and normal groups was greater than the variation between rats. Therefore, the two experiments were pooled to arrive at the final rates of absorption which were

TABLE 3  
ABSORPTION OF HISTIDINE IN NORMAL AND INFESTED RATS

Rat No.	Normal Rats		Infested Rats		
	Body Weight (g)	Absorption (mg/40 min)	Body Weight (g)	Absorption (mg/40 min)	Worm Counts
1	154	103	169	70	2000
2	153	115	195	106	1000
3	166	124	186	115	1400
4	182	107	180	143	1500
5	158	130	135	131	1600
6	161	123	162	127	1300
7	154	122	168	88	900
8	153	134	182	132	500
9	148	119	127	104	900
10	—	—	137	117	2000
11	—	—	155	122	2000
Mean	159 $\pm$ 10.2	120 $\pm$ 10.1	163 $\pm$ 22.5	114 $\pm$ 21.0	

then the mean values for 12 rats each. These are shown in Table 5 and indicate clearly that the rate of absorption per gram dry mucosal tissue per hour from the jejunum of infested animals is reduced to about a quarter or a fifth of that in the normal rat.

This can be compared with the rate of efflux of sodium which was reduced to a third of the normal rate in the earlier experiment (Symons 1960*b*). Also included in Table 5 are the rates of absorption expressed as  $\mu$ moles per centimetre length of the jejunal loops. The reasons for expressing the rates in these two terms has already been discussed in Part II (Symons 1960*a*). It shows that the greater surface area per centimetre of infested intestine partly compensates for the lower rate of absorption per unit of mucosal tissue.

These results can be compared with those of Fullerton and Parsons (1956) who perfused the intestine by means of a recirculating unit with various concentrations of glucose in bicarbonate buffer. It has been estimated by Symons (1960*a*)

that in normal rats the dry jejunal tissue weighs 0.021 g per centimetre length. The rate of absorption of 59  $\mu$ moles/hr/cm found in these experiments then becomes 2.8  $\mu$ moles/mg/hr from a solution containing 250 m-moles glucose/l. The rates recorded by Fullerton and Parsons were 4.4  $\mu$ moles/mg dry weight/hr from a solution containing 246 m-moles/l and 3.1  $\mu$ moles/mg/hr from a solution of 56 m-moles/l. The rate for this experiment was therefore a little lower but this may be because Fullerton and Parsons used a perfusing solution which was adjusted to about isotonicity whereas the solution used in this experiment was hypertonic and was accompanied by a net influx of water during perfusion.

TABLE 4  
PERCENTAGE OF FED GLUCOSE AND HISTIDINE ENTERING THE SMALL INTESTINE  
OF NORMAL AND INFESTED RATS IN 40 MIN

Rat No.	Glucose (%)		Histidine (%)	
	Normal Rats	Infested Rats	Normal Rats	Infested Rats
1	48.7	79.2	80.9	98.4
2	74.0	83.3	84.6	81.8
3	78.3	61.1	89.7	95.0
4	75.7	47.2	86.0	93.6
5	43.3	79.2	89.1	94.1
6	65.2	78.9	87.0	93.4
7	59.8	77.5	94.1	73.5
8	58.7	74.6	86.2	95.6
9	52.8	63.4	84.7	92.5
10	77.4	72.6	—	85.9
11	56.4	—	—	84.1
Mean	62.8 $\pm$ 12.2	71.7 $\pm$ 11.2	86.9 $\pm$ 3.7	89.8 $\pm$ 7.5

#### IV. DISCUSSION

First, it is necessary to comment upon the rates of absorption from the small intestine as a whole. The rate of glucose absorption agrees reasonably well with those reported by Fenton (1945) and Heller (1954) who used similar concentrations and time intervals. No strictly comparable results could be found for absorption of histidine, but Hird and Sidhu (1957) have measured its rate of absorption in perfusion experiments. Their values of about 330  $\mu$ moles/40 min/g dry weight of small intestine can be converted to 110 mg/40 min assuming that approximately 80 cm of intestine are effectively involved in these experiments and that 1 cm contains 0.021 g dry weight. This value compares favourably with that of 120 mg/40 min for normal rats shown in Table 3.

Fenton (1945) and Reynell and Spray (1956) have discussed gastric absorption of glucose. The latter found that there was some small but undetermined absorption from the stomach of the rat provided that it was not ligated at the pylorus. The

results of the experiments reported here include any glucose or histidine absorbed by that organ, but as the rate of gastric emptying was the same for both normal and infested rats it is unlikely that there would be any difference in gastric absorption between the two groups.

No explanation can be offered for the fact that there was a correlation between gastric emptying and glucose absorption but not for gastric emptying and histidine absorption. It may be that the variation of gastric emptying rate between individual rats was not sufficiently great as regards histidine to indicate a relationship with absorption.

It is clear that the infestation did not affect the absorption of glucose or histidine from the small intestine as a whole. On the other hand, glucose absorption from the jejunum was severely affected; in fact, it was reduced to less than a quarter of the rate per gram of dry mucosal tissue in the normal rat. It has already

TABLE 5  
GLUCOSE ABSORPTION FROM THE JEJUNA OF NORMAL AND INFESTED RATS PERFUSED  
IN VIVO  
Glucose concentration in perfusion solution 0.25M

	Absorption Rate (m-moles/hr/g dry mucosal tissue)		Absorption Rate ( $\mu$ moles/hr/cm length jejunum)
Normal rats	$3.92 \pm 2.12$	$\left. \begin{array}{c} \\ \end{array} \right\} P < 0.01$	$59 \pm 32$
Infested rats	$0.88 \pm 1.02$		$20 \pm 23$

been explained that this expresses the rate of absorption in terms of unit functional tissue. The reduction of absorption is not quite so striking when expressed in terms of length of intestine because of the greater weight of mucosa per unit length in the infested rat (Symons 1960a).

This derangement of absorption in the jejunum supports the results of the earlier experiment of Symons (1960b) when it was found that the rate of sodium efflux from the lumen is reduced to about one-third by the infestation.

At this stage it is not possible to account for the contradiction between glucose absorption from the jejunum alone and from the entire small intestine. It can be postulated that some compensatory mechanism is present, or at least that there is no derangement of the function of the non-infested parts of the small intestine. Only perfusion of these sections of the gut can settle this question.

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# SOME FACTORS INFLUENCING THE BREAKDOWN OF CELLULOSE BY BACTERIA

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## Summary

Variations in the extent of breakdown of celluloses from different sources by *Cellulomonas biazotea* can be accounted for in terms of the crystallinity and content of associated pentosan of the celluloses. Under similar conditions, *Sporocytophaga myxococcoides* did not distinguish between celluloses and degraded the different materials at the same rate.

## I. INTRODUCTION

A number of workers have studied the factors influencing the rate of microbiological breakdown of cellulose with varied and sometimes contradictory results. Hajo (1942) concluded that the resistance of cellulose to attack by a number of organisms depended on its chain length; Fuller and Norman (1942) found no difference in the growth rate of a number of bacteria on a series of cellodextrins having various chain lengths; and Basu and Ghose (1952) found that a hydro-cellulose prepared by hydrolysing jute was more resistant than the original cellulose.

The greatest difficulty in correlating these varied observations lies in the fact that different workers have used different organisms and also cellulose from different sources and of different past histories. Furthermore, most workers have attempted to study the effect of changes in only one property of the cellulose at a time. While this would be highly desirable, if it were possible, the properties of cellulose are not independent variables and it is not, in general, possible to alter one without changing others. For example, attempts to prepare a series of celluloses of differing chain length by acid hydrolysis will result in a series of materials which also have differing crystallinities, depending on the time of hydrolysis and the extent to which the crystalline and amorphous regions of the cellulose have been attacked (Nickerson 1950). Consequently it will not be possible to decide whether the effects observed are due solely to changes in chain length or to what extent they are also influenced by the concomitant changes in crystallinity.

In the present investigation a number of samples of  $\alpha$ -cellulose have been prepared from a variety of sources and determinations made of those properties which it was thought might influence the rate of bacterial breakdown. An attempt has then been made to correlate variations in these properties with variations in the rate of breakdown, and for this purpose the technique of multiple regression analysis has been used to estimate the effect produced by variation in one property of the cellulose at a time.

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## II. MATERIALS AND METHODS

(a) *Culture Conditions*

The organisms used were a strain of *Cellulomonas biazotea*, obtained from the Department of Bacteriology, University of Queensland, and *Sporocytophaga myxococcoides*, ATCC 10011. They were maintained on slopes of the following composition:

	g/l		g/l
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2.5	FeNH <sub>4</sub> (SO <sub>4</sub> ) <sub>2</sub> ·12H <sub>2</sub> O	0.01
K <sub>2</sub> HPO <sub>4</sub>	1.0	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.002
NaCl	1.0	MnSO <sub>4</sub> ·7H <sub>2</sub> O	0.001
MgSO <sub>4</sub> ·7H <sub>2</sub> O	1.0	Difco yeast extract	0.01
CaCl <sub>2</sub>	0.02	Agar	15.0

to which, for the *Cellulomonas*, 20 g/l of sucrose were added and, for the *Sporocytophaga*, a strip of filter paper was laid on the surface. The experiments on the rate of cellulose breakdown were carried out in shake cultures as described previously (Youatt 1958) using 50 ml of the above liquid medium and adding 100 mg of the cellulose to be examined. Incubation was at 28°C.

(b) *Preparation of Cellulose Samples*

The procedure for isolating the  $\alpha$ -celluloses was based on that given by Jermyn (1955). Fresh plant material was first killed by immersion in 4 volumes of boiling ethanol. After air drying it was ground in a Wiley mill to pass a 60-mesh screen and extracted with benzene-ethanol azeotrope for 8 hr. This material was further extracted with a large volume of water in a boiling water-bath for 8 hr and, following treatment with sodium chlorite using the method of Wise, Murphy, and D'Addieco (1946), the resulting holocellulose was extracted with 17.5 per cent. sodium hydroxide for 1 hr in stoppered flasks with the minimum amount of ullage. The  $\alpha$ -cellulose was filtered, washed with 4 per cent. sodium hydroxide, water, 10 per cent. acetic acid, water again, and finally with ethanol and acetone and air dried. Eucalypt "high  $\alpha$ " pulp and Whatman No. 541 paper samples were used without further treatment.

(c) *Determination of Residual Cellulose*

The determination of the cellulose remaining in the cultures was based on the method given by Dorée (1947). The entire culture was centrifuged, the supernatant removed as completely as possible, and the residue, consisting of bacteria and undigested cellulose, treated with 10 ml of 17.5 per cent. sodium hydroxide. After standing, with occasional shaking, for half an hour the cellulose was centrifuged again, washed with water, and dissolved in 15 ml of 72 per cent. sulphuric acid. 10 ml of 10 per cent. potassium dichromate was added and the solution heated for 20 min at 80°C. After cooling the excess dichromate was titrated with ferrous ammonium sulphate. In some experiments, where there were only a few estimations to be made, the cellulose, after treatment with sodium hydroxide, was collected on a sintered-glass crucible, washed well, dried at 105°C for 2 hr, and weighed.

(d) *Determination of Moisture Regain*

The moisture regains were determined on samples of cellulose which had been autoclaved under the same conditions as those used when preparing the bacterial cultures. After autoclaving the cellulose was filtered from the salts solution, washed well, and freeze-dried. It was then conditioned at 20°C and 65 per cent. R.H. for at least 24 hr, weighed, dried at 105°C for 2 hr, and reweighed.

TABLE 1  
BREAKDOWN OF CELLULOSE IN CELLULOMONAS CULTURES

Source of Cellulose	Residual Cellulose (mg)			
	Period of Incubation (days)			
	0	1	2	3
Cotton	95.6	94.8	92.9	90.4
Rye grass	72.9	67.7	58.6	56.9
<i>Phalaris</i>	83.2	82.3	76.8	70.5
Eucalypt $\alpha$ pulp	73.6	69.6	67.3	68.8
Carnation stem	84.3	70.5	75.3	67.4
Potato tuber	61.4	58.8	44.5	36.7
Flax	97.2	94.9	81.6	89.9
Pea pod	78.7	67.7	47.6	47.9
Hemp	89.4	87.1	79.5	78.1
<i>Eucalyptus rostrata</i>	87.6	90.7	80.5	80.2
Ivy leaves	76.3	76.2	66.1	53.1
Cabbage leaves	79.3	68.1	52.8	57.6
Carrot phloem	63.5	61.2	51.4	45.4
Kenaf	80.7	86.1	81.4	77.3
Whatman paper No. 541	92.5	92.1	91.5	90.7

(e) *Determination of Chain Length*

The Swedish Association of Pulp and Paper Engineers cupriethylenediamine method (1957) was used. Ostwald viscometers were employed and no corrections for velocity gradient were made as the relative order rather than the absolute value of the chain lengths was sufficient for the present purpose.

(f) *Determination of Pentosan*

The pentosan remaining in the  $\alpha$ -cellulose was determined by the method of Mackney and Reynolds (1938).

(g) *Determination of Residual Lignin*

The lignin content of the  $\alpha$ -celluloses was estimated from measurements of the ultraviolet absorption of solutions of the cellulose in 72 per cent. sulphuric acid, using as a standard a sample of eucalypt "methanol" lignin (Bland *et al.* 1947).

*(h) Estimation of Crystallinity and Extent of Oxidation from Infra-red Spectra*

The infra-red spectra were obtained with the sample in the form of pressed KCl disks. As it is not possible to obtain an unequivocal quantitative measure of crystallinity for a series of samples from such diverse sources, the materials were examined and arranged in decreasing order of crystallinity by a subjective appraisal of the sharpness of the spectra. A similar procedure, based on the intensity of carbonyl absorption at  $5.8 \mu$ , was used for evidence of oxidation of the sample.

## III. RESULTS

Table 1 shows the amount of cellulose remaining in cultures after the growth of *Cellulomonas* for periods of up to 3 days. This short period was chosen in the hope that any effects of variations in the properties of the cellulose samples would

TABLE 2  
PROPERTIES OF CELLULOSE PREPARATIONS

Source of Cellulose	Moisture Regain (%)	Residual Pentosan (%)	Chain Length	Residual Lignin (%)	Nitrogen Content (%)
Cotton	10.5	0.6	1700*	0.10	0.01
Rye grass	11.0	5.1	716	0.44	0.14
<i>Phalaris</i>	9.7	3.0	852	0.40	n.d.†
Eucalypt a pulp	8.1	1.0	359	0.12	n.d.
Carnation stem	8.6	1.5	629	0.78	n.d.
Potato tuber	12.1	3.0	693	0.38	0.15
Flax	9.2	0.1	1625	0.13	0.01
Pea pod	12.7	18.0	840	0.57	0.15
Hemp	9.6	1.0	1098	0.58	0.01
<i>E. rostrata</i>	11.3	5.5	928	1.49	n.d.
Ivy leaves	10.4	8.6	444	0.43	0.41
Cabbage leaves	11.8	6.7	440	0.13	0.36
Carrot phloem	15.9	11.7	621	0.07	0.11
Kenaf	11.3	1.9	818	1.55	0.02
Whatman paper No. 541	7.7	0.3	957	0.01	n.d.

\* This material was not completely soluble in cupriethylenediamine and the value given is therefore a lower limit.

† n.d., not detectable.

be most marked in the initial stages of the attack. Three replicates were used to estimate the linear breakdown rate for each material. There was no evidence for non-linearity in the breakdown rates and a study was made by means of multiple regression of the relation between breakdown rate ( $y$ ) and the properties of the celluloses listed in Tables 2 and 3.

The regression coefficients associated with chain length and lignin content were not significant, but the remainder were significant at the following levels:



regain ( $x_1$ )  $P < 0.02$ , pentosan content ( $x_2$ )  $P < 0.001$ , and infra-red crystallinity order ( $x_3$ )  $P < 0.001$ . The regression equation is

$$y = 3.33 - 0.38x_1 + 0.19x_2 + 0.29x_3.$$

As a check on the adequacy of this equation two further samples of cellulose were prepared from mint and clover leaves. Determinations were made of their

TABLE 3  
CHARACTERISTICS OF CELLULOSE SAMPLES DERIVED FROM INFRA-RED SPECTRA

Source of Cellulose	Descending Order of Crystallinity	Decreasing Extent of Oxidation
Whatman paper No. 541	1	5
Eucalypt $\alpha$ pulp	2	
<i>E. rostrata</i>	3	
Carnation stem	4	
Kenaf	5	
Cotton	6	
Flax	7	7 (slight)
Hemp	8	
<i>Phalaris</i>	9	
Rye grass	10	
Ivy leaves	11	1
Pea pod	12	6
Cabbage leaves	13	2
Potato tuber	14	2
Carrot phloem	15	2

moisture regain, pentosan content, and infra-red spectrum and a comparison made between the observed rates of breakdown by *Cellulomonas* and those predicted by the above equation. The results are given in Table 4.

TABLE 4  
COMPARISON OF PREDICTED AND OBSERVED RATES OF BREAKDOWN OF CELLULOSE  
IN CELLULOMONAS CULTURES

Source of Cellulose	Moisture Regain (%)	Residual Pentosan (%)	Infra-red Crystallinity Order	Predicted Breakdown (mg/day)	Observed Breakdown (mg/day)
Clover	11.2	5.4	7-8	2.82	2.38
Mint	14.4	9.2	12-13	4.10	3.75

Since the pentosan content of the  $\alpha$ -celluloses had such a marked effect on their rate of breakdown an examination was made of the effect of additions of

extra pentosan to the bacterial cultures. Table 5 shows the effect of added hemicellulose on the extent of breakdown of the corresponding cellulose. The hemicellulose fractions were prepared by precipitating the 17.5 per cent. sodium hydroxide extracts obtained in the preparation of  $\alpha$ -cellulose with acidified ethanol and contained a high proportion of pentosan.

Table 6 shows the results for the breakdown of cellulose in cultures of *Sporocytophaga*. These results differ markedly from those obtained with *Cellulomonas* for there is no significant difference between the extent of breakdown of the various cellulose samples.

TABLE 5  
EFFECT OF ADDED PENTOSAN ON THE EXTENT OF CELLULOSE  
BREAKDOWN BY CELLULOMONAS

Source of Cellulose	Cellulose Degraded in 3 Days (mg)	
	Control Cultures	Plus 50 mg Hemicellulose
Flax	6.0	6.4
	9.1	5.8
<i>Phalaris</i>	20.3	21.3
	22.3	18.9

#### IV. DISCUSSION

In recent years much information has accumulated about enzymes capable of splitting soluble or degraded  $\beta$ -1,4-glucan chains, but there is still very little understanding of the initial steps in the breakdown of insoluble cellulose.

The satisfactory agreement between the observed rates of breakdown of clover and mint leaf celluloses by *Cellulomonas* and those predicted from the equation (Table 4) indicate that the correlations found account for the major factors influencing this organism.

The most significant correlation is with the infra-red "crystallinity" order. Forziati and Rowen (1951) have shown that the increasing loss of sharpness in the infra-red spectrum is associated with the change from cellulose I through cellulose II to amorphous cellulose. Hence it seems reasonable to deduce that lack of crystalline organization is the major property of cellulose influencing the attack by this organism. Such an interpretation is in keeping with the results of other workers (Norkrans 1950; Walseth 1952).

The moisture regain of cellulose depends on the crystallinity and the swelling. Owing to the nature of the statistical analysis employed, it is believed that, in this case, the effect of crystallinity has already been accounted for in the correlation with the infra-red crystallinity order and that the correlation with moisture

regain refers predominantly to the effect of the swelling introduced during the preparation of the samples. Ant-Wuorinen and Vispää (1959) have shown that the swelling of cellulose frequently decreases its accessibility. On this basis the negative correlation of rate of breakdown with moisture regain is readily understandable. The conventional methods of swelling cellulose for use in bacterial media or enzyme experiments are successful, therefore, because the effect of diminished crystallinity far outweighs the effect of diminished accessibility due to swelling.

TABLE 6  
BREAKDOWN OF CELLULOSE IN SPOROCYTOPHAGA CULTURES

Source of Cellulose	Residual Cellulose (mg)			
	Period of Incubation (days)			
	0	1	2	3
Cotton	91.5	84.7	88.9	80.0
	96.1			89.7
Rye grass	79.1	75.0	74.9	61.7
	84.2			74.4
<i>Phalaris</i>	81.0			69.7
Eucalypt $\alpha$ pulp	78.4	72.8	74.8	50.6
	90.9			85.1
Flax	94.1	88.6	91.2	84.4
	92.1			89.2
Pea pod	71.4	71.2	70.1	52.8
	70.4			55.0
Hemp	85.9	84.9	82.9	75.7
	93.2			88.9
Ivy leaves	62.0	63.1	59.7	58.0
Carrot phloem	62.7	60.7	56.6	49.6
	66.4			62.1
Kenaf	85.8	80.5	80.9	70.7
	90.7			83.3
Whatman paper No. 541	90.0	94.7	90.6	79.0
	100.0			96.8

Since *Cellulomonas* grows readily on pentosans it might be thought that the role of residual pentosan was to provide an additional carbon source for the growth of the organism. This explanation appears to be invalidated by the fact that addition of pentosan did not diminish the rate of cellulose breakdown (Table 5). An alternative possibility is that the presence of pentosan in the  $\alpha$ -cellulose constitutes, in some way, a structural defect such that its removal in the course of bacterial growth renders the cellulose more available to the organism. However, in view of the lack of knowledge of the way in which non-glucose polysaccharides are associated with  $\alpha$ -cellulose this cannot be more than speculation.

The failure to demonstrate any response of *Sporocytophaga* to changes in cellulose properties is in direct contrast to the experience with *Cellulomonas*. The experimental variability with the two organisms was of the same order and it must be concluded that if *Sporocytophaga* is affected by changes in cellulose properties then these effects are only slight and different preparations are degraded at essentially the same rate. Reese and his co-workers (Reese 1956) have recently separated the cellulase of *Trichoderma viride* into a number of components and have shown differences in specificity between them. If the proportions between the components of a cellulase system are capable of adaptive change in response to variations in substrate properties then no effect in the overall response of the organism would be expected. Such a difference in the behaviour of two organisms strongly underlines the dangers of any attempt to deduce a composite theory of cellulose breakdown by the combination of results obtained with different organisms and different substrates.

#### V. ACKNOWLEDGMENTS

The author is extremely grateful to Mr. W. B. Hall for the statistical work required in this paper, to Mr. H. Higgins for the infra-red spectra, and to Mr. B. W. Wilson for technical assistance. The sample of eucalypt lignin was kindly supplied by Mr. A. J. Watson.

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## SHORT COMMUNICATIONS

### THE EFFECTS OF INJECTED BIOTIN AND DETHIOBIOTIN ON BUTTER YELLOW CARCINOGENESIS IN RATS FED A PROTECTIVE DIET\*

By M. H. BRIGGS†

Following the discovery of the carcinogenicity of the azo dye butter yellow (*N,N*-dimethyl-*p*-aminoazobenzene), a number of investigators (Nakahara, Mori, and Fujiwara 1938*a*, 1938*b*) established that feeding yeast or liver extracts to rats afforded them considerable protection against tumor formation by the dye. Working with purified diets, two further groups (Gyorgy, Poling, and Goldblatt 1941; Kensler *et al.* 1941) demonstrated that excess casein and riboflavin together were also highly protective against the action of the dye. However, du Vigneaud *et al.* (1942) have reported that the daily addition of 2  $\mu$ g of crystalline biotin to such a diet destroys its protection. This finding was confirmed in more extensive experiments by Burk *et al.* (1943), though Kline, Miller, and Rusch (1945) demonstrated that the dietary casein could be replaced by egg white and injections of 6  $\mu$ g of biotin weekly without affecting the protection.

More recently Axelrod and Hofmann (1953), using a protective diet similar to the previous studies, were unable to demonstrate any procarcinogenic action by either biotin or oxybiotin. The present experiments were therefore undertaken to investigate the effects of biotin and dethiobiotin injections on rats fed a protective diet containing butter yellow.

#### *Experimental*

Thirty-six male albino weanling rats of the Sprague-Dawley strain were obtained from Holtzman Inc. and were singly caged in wide-mesh, screen-bottomed cages. For the first 4 weeks they were fed the basal diet (see Table 1) without injections. After this time the animals were divided into three equal groups of 12 rats. Group 1 received injections of 5  $\mu$ g of *d*-biotin weekly; group 2 received 50  $\mu$ g of *d*-biotin, while group 3 received 50  $\mu$ g of *d*-dethiobiotin. All solutions were prepared in sterile saline and injections were given subcutaneously twice weekly in two equal doses.

Throughout the experiment the rats were frequently weighed and the consumption of food and water (which were fed *ad libitum*) was determined every 2 weeks. The mean weight of each group at the beginning of the experiment was 66 g. At the end of the experiment the mean weights were: group 1, 225 g; group 2, 232 g; and group 3, 189 g. The growth on dethiobiotin is greater than would have been predicted from the results of Rubin, Drekter, and Moyer (1945).

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After 140 days from the beginning of the injections all animals were still alive and apparently healthy, though group 2 animals had brown pigment in the fur on their heads and backs. No tumors were detectable by palpation. The animals were killed and an immediate post-mortem macroscopic examination was made of

TABLE 1  
COMPOSITION OF THE BASAL DIET

Constituent	Concentration (g/100 g diet)	Constituent	Concentration (g/100 g diet)
Vitamin-free casein	15	Pyridoxine	0.0005
Dried egg white	10	Nicotinic acid	0.002
"Crisco"	10	<i>i</i> -Inositol	0.1
Sucrose	60	Calcium pantothenate	0.001
L-Cystine	1	2-Methyl-1,4- naphthoquinone	0.0001
Osborne-Mendel salt mixture	4	<i>dl</i> - $\alpha$ -Tocopherol acetate	0.001
Choline	0.25	Butter yellow	0.1
Riboflavin	0.002	Vitamin A	4070 i.u.
Thiamine	0.0005	Vitamin D	814 i.u.

all major organs. The liver and kidneys of each animal were removed and half of each organ preserved for microscopic examination. The other half was assayed for total biotin by the *Lactobacillus arabinosus* method of Wright and Skeggs (1944) and also the yeast method of Hertz (1943).

TABLE 2  
BIOTIN CONTENTS OF RAT TISSUES

Group	Tissue	Total Biotin* ( $\mu$ g/g fresh tissue)
1	Liver	0.53
	Kidney	0.29
2	Liver	0.59
	Kidney	0.30
3	Liver	0.51
	Kidney	0.26

\*Mean of 12 determinations.

### Results

No tumors were discovered in any animal on macroscopic or microscopic examination. No other abnormalities of any type were found, though cirrhosis and bile-duct hyperplasia were particularly looked for. The biotin contents of the

livers and kidneys of each group are given in Table 2. The values recorded are derived from the yeast assay which consistently gave biotin values about 5 per cent. higher than the *L. arabinosus* assay. These biotin values are comparable with those of normal organs and are much higher than the biotin contents of butter yellow hepatomas reported by Pollack *et al.* (1942) and West and Woglom (1942).

The present experiment demonstrates that neither biotin nor dethiobiotin, injected at the levels stated above, can destroy the protection against butter yellow carcinogenesis afforded to rats by dietary casein and riboflavin supplements. The difference between this result, together with that of Axelrod and Hofmann (1953), and the earlier findings may be due to variations in the purity of the dietary constituents.

#### *Acknowledgment*

This work was carried out largely at the Department of Biochemistry and Nutrition, Cornell University, Ithaca, New York.

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## THE EXPERIMENTAL PRODUCTION OF EPITHELIUM-LINED CYSTS\*

By G. S. MOLYNEUX†

In a previous communication (Molyneux 1959) the bacterial degradation of wool fibres *in vitro* was described. The causative organism, an aerobic mesophilic spore-forming rod, was isolated from the contents of epithelium-lined cysts produced experimentally in sheep. This paper describes the production of these cysts by a technique which was developed in order to study the mechanism of cyst growth.

\* Manuscript received September 30, 1959.

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The implantation technique involves the splitting of skin between the reticular and hypodermal layers of the dermis while cyst formation is ensured by maintaining the blood supply to the implant. The epithelium-lined cysts produced by this technique satisfy the following requirements:

- (1) Success of cyst formation.
- (2) Controlled initial size of the cyst.
- (3) Uniformity of cyst wall structure.
- (4) Maximum fluidity of cyst contents.
- (5) Maximum survival time of an intact epithelial lining.

Preliminary studies indicated that the above requirements for an experimental cyst were obligatory, if the mechanisms of cyst growth were to be adequately studied. Davis and Traut (1926) and Butcher (1946) formed epithelium-lined sacs by implanting full thickness skin in dogs and rats respectively. In both these studies, although cyst formation occurred, the cyst wall was not uniform in structure, the epithelium and cyst wall always being thicker over the area of the original graft. Because it was intended to make biopsies of the cyst wall at intervals, in order to study the relation between the increase in cyst size and histological changes observed in the epidermis and its derivatives, uniformity in cyst wall structure was essential. This uniformity has been achieved in the developed technique as shown in Plate 1, Figure 1. The cyst wall consists of full thickness skin and panniculus carnosus muscle, except at the area of closure (*A*) from which muscle, hair follicles, and sebaceous glands are absent.

The technique was developed in rabbits but was used in sheep (an animal provided with sweat glands) in order to increase the fluid content of the cysts so that intracystic pressures could be recorded.

### *Experimental*

The technique for the production of epithelium-lined cysts was developed in albino rabbits of approximately 1.5 kg weight. The animals were anaesthetized with "Nembutal" given parenterally into the lateral ear vein. The mid-side skin of the animal was prepared by close clipping, defatting with ether and alcohol, washing in soap and water, and then applying "Cetavlon".

Circular areas 4 cm in diameter were outlined with gentian violet on skin overlying the lateral aspect of the thorax. An initial incision was made through the full thickness of the skin (extending from *A* to *B*, Fig. 1(*a*)). A curved incision *BCD* was then made through the epidermis and into the dermis to a level where, as described by Medawar (1944), the skin splits from its substratum. Medawar (1944), in describing the structure of rabbit skin, says: "The principal part of the dermis which underlies it [the epidermis], 0.40–2.00 mm in thickness, is composed of stout collagen fibres in the typical three-dimensional packing of compact connective tissue [reticular layer]. It grades superficially into the papillary layer, where the fibres are smaller and in more open packing; and below, rather sharply, into a layer where the fibres are orientated two-dimensionally in the plane of the integument [hypodermis]. At this deeper level, therefore, the skin 'splits' naturally from its substratum. The



principal arteries, veins, lymphatics and nerves of the integument travel in the fascial [hypodermal] layer, and they likewise run in the plane of the integument." Thus the incision *BCD* into the dermis was extended to a level between the reticular and fascial layers as shown by *S* in Plate 1, Figure 2. The principal vessels and nerves are contained in the deeper fascial layer which overlies the panniculus carnosus muscle. The incision was then extended from *D* to *F* through the full thickness of the skin, as for the incision from *A* to *B*.

Commencing at *C* the dermis was then split in the direction *GH* in the plane between the reticular and fascial layers. The splitting was continued until it covered the area indicated by the broken lines *BG* and *DH* shown in Figure 1(b). This distance should be sufficient to enable the eventual implant *BCDE* to be drawn away from the proximity of the original suture line *ABDF*. The dissection frees from

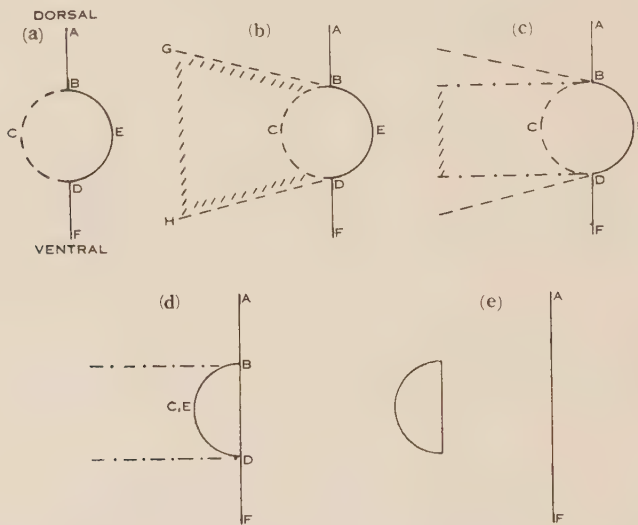


Fig. 1.—Diagram illustrating the method of preparing and placing the implants (see text for further explanation).

the fascial layer the epidermis and superficial dermis, leaving them attached to the fascial layer and blood supply along a perimeter indicated by the shaded area. An incision along the curve *BED* was then made through the full thickness of the skin. The skin to be implanted, *BCDE*, originally marked in gentian violet, can then be freed by blunt dissection from the loose connective tissue overlying the panniculus carnosus. This leaves it still attached to a vascular fascial pedicle, *BCDHG*, actually shown in Plate 1, Figure 2. This skin can now be lifted free from the body wall. To free the implant further, making it freely moveable, the fascial layer was divided\* as indicated in Figure 1(c), resulting in a narrowing of the base of the fascial pedicle.

\* If this division is attempted earlier, difficulty will be experienced in stabilizing the implant during dissection.

The skin was then folded along the line *BD*, the free edges of the two semicircles *BED* and *BCD* being carefully apposed and sutured with fine non-chromic catgut. A closed semicircular sac lined by surface epithelium was thus formed (Fig. 1(*d*)). The implant can be transplanted either to a position between the reticular and fascial layers (area *BCDHG* in Fig. 1(*b*)) or beneath the fascial layer above the panniculus carnosus (by splitting this area in the deeper plane). In either of these positions it can be secured by a suture. The implant was placed laterally to the original suture line *AF* in order to minimize the tracking of infection from the surface to the cyst cavity.

To close the gap left by removal of the implant the surrounding tissues were freed by blunt dissection, the wound edges trimmed then closed in layers. The final result is indicated in Figure 1(*e*). This technique was then applied to sheep and seven implants were made in Merino and Corriedale sheep.

#### *Examination of Cysts*

The area of skin covering and surrounding the cyst was closely clipped as in Plate 2, Figure 1, and the cyst size measured with calipers. At implantation the cysts were semicircular but tended to become spherical during growth. The measurements, because they included the thickness of the surface skin, gave an indication of growth rather than an accurate record of size.

Biopsy was performed by the following method: An incision was made at a position adjacent to the cyst to the level of the panniculus carnosus (depending on the original depth at which the cyst had been implanted). The cyst was exposed by dissection, and by freeing the surrounding tissue the undersurface of the cyst could be turned towards the surface. The cyst was held firmly in this position while a biopsy punch 0.5 cm in diameter, similar to that used by Carter and Clarke (1957), was used to take a sample of the cyst wall (Plate 3). A biopsy punch 1 cm in diameter was used to take a control sample of surface skin adjacent to the cyst. Intracystic wool samples and the aspirated fluid contents of each cyst were also collected and examined.

#### *Results*

Seven implants were made in Merino and Corriedale sheep. Early cyst formation resulted in each case. Because of the presence of sweat glands, which remain active and apparently excrete into the cyst, the success of the implant can be judged within 10 days by the distention of the experimental cyst. There may be a large amount of fluid in the cyst; in one instance 12 ml of a brown clear fluid were aspirated. At intervals samples of cyst content, fluid and wool, were taken and biopsies of the cyst wall were made.

An example of an experimental cyst removed 10 months after implantation is shown in Plate 2, Figure 2. At formation the implant consisted of a semicircular (approx. 4 by 2 cm) double thickness layer of skin. At removal the cyst measured 6.5 by 4 cm. Because the cyst wall was uniform in structure, sampling by biopsy was possible and because of the thickness of sheep skin the dissection and fabrication of cysts were technically easier than with rat or rabbit skin, allowing a more accurate apposition of the graft-cut surfaces.

### Acknowledgments

Through the courtesy of Dr. D. F. Stewart, McMaster Animal Health Laboratory, C.S.I.R.O., Glebe, N.S.W., and of Dr. I. W. McDonald, Sheep Biology Laboratory, C.S.I.R.O., Prospect, N.S.W., facilities and animals were made available for the experimental work.

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## EXPLANATION OF PLATES 1-3

### PLATE 1

- Fig. 1.—Experimental cyst from rabbit skin with attached blood supply as seen after 7 weeks. *A*, area of surgical closure. Hair follicles are absent.
- Fig. 2.—Longitudinal section showing the full thickness of rabbit skin indicating the planes of surgical division. *ep*, epidermis; *dm*, dermis (papillary and reticular layers); *hy*, hypodermis containing blood vessels; *pc*, panniculus carnosus muscle; *S*, superficial plane of division for area *BCDHG* (Fig. 1(b)); *D*, deep plane of division for area *BCDE* (Fig. 1(c)).

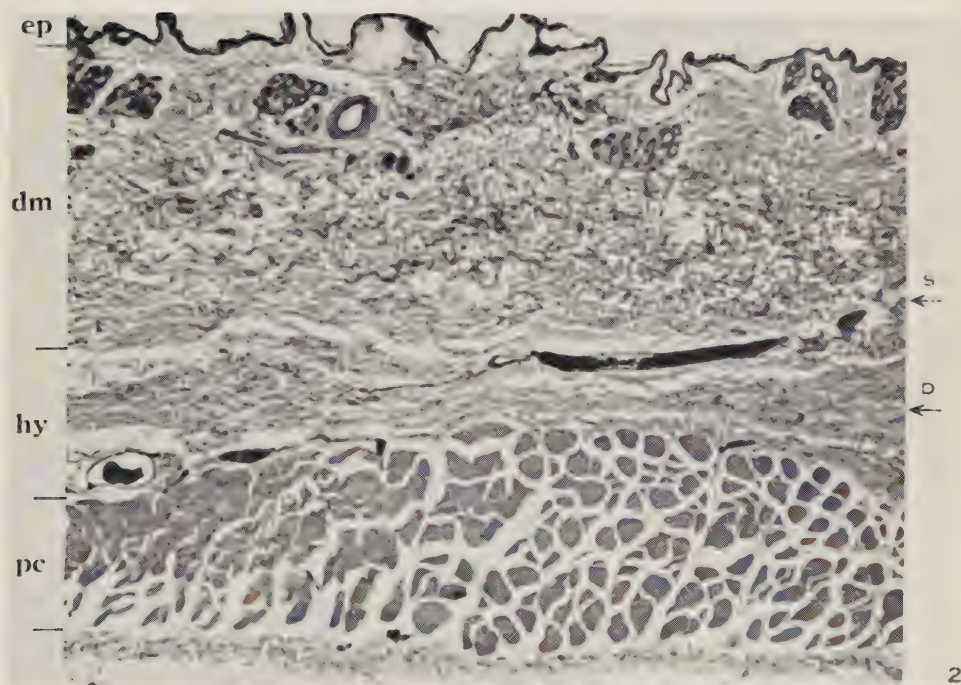
### PLATE 2

- Fig. 1.—Mid-side of the trunk of a Merino sheep showing the position of three implanted experimental cysts.
- Fig. 2.—Experimental cyst from sheep skin with attached blood supply. The surface skin was left attached. This is the anterior cyst from animal shown in Plate 2, Figure 1.

### PLATE 3

- Fig. 1.—Experimental cyst from Merino sheep skin. Inverted cyst showing biopsy punch in position.
- Fig. 2.—Biopsy freed from the cyst wall but still attached to the intracystic wool.
- Fig. 3.—Biopsy completed and the tissue removed.

EXPERIMENTAL PRODUCTION OF EPITHELIUM-LINED CYSTS

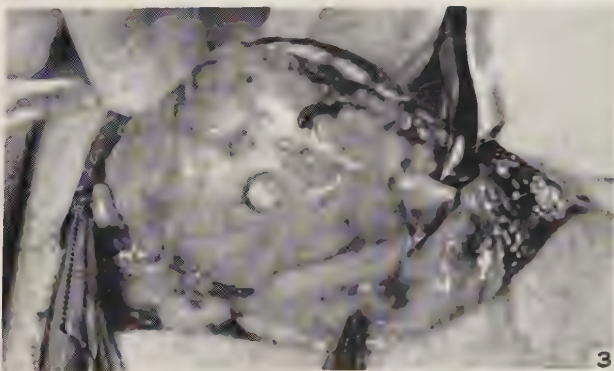




EXPERIMENTAL PRODUCTION OF EPITHELIUM-LINED CYSTS



EXPERIMENTAL PRODUCTION OF EPITHELIUM-LINED CYSTS





# THE PHYSIOLOGY OF SUGAR-CANE

## III. CHARACTERISTICS OF SUGAR UPTAKE IN SLICES OF MATURE AND IMMATURE STORAGE TISSUE

By R. L. BIELESKI\*

[*Manuscript received February 8, 1960*]

### *Summary*

Sugar uptake by slices of sugar-cane storage tissue took place in two stages. The initial uptake reached an equilibrium within 1 hr, the level being proportional to the external sugar concentration, independent of the sugar, and unaffected by anaerobic conditions. This sugar diffused out rapidly when the tissue was placed in water. It was thus contained in the apparent free space, 10–20 per cent. of the tissue volume. The secondary uptake continued up to 60 hr at a slow, constant rate, 1–5 mg/g/day, independent of sugar concentration above 2.0 per cent., dependent on the sugar, and inhibited by anaerobic conditions. This sugar did not diffuse out when the tissue was placed in water. It was concluded that the secondary uptake was an active accumulation process.

The sucrose content of the tissue increased during accumulation, which occurred against a 10- to 200-fold concentration gradient. There was no starch synthesis, but accumulation was associated with a 30–40 per cent. increase in respiration. Sucrose was not hydrolysed prior to accumulation, and when slices accumulated sugar from a mixed solution, sucrose uptake inhibited glucose uptake. Internodes which were most active in storing sugar in the field gave the most actively accumulating preparations in laboratory experiments. Mature tissue slices showed little or no ability to accumulate sugars.

### I. INTRODUCTION

Sugar transport† in animal tissues (LeFevre 1954, 1955) and sugar accumulation in bacteria (Davis 1956; Monod 1956) and yeast cells (Rothstein 1954) are well-documented processes. Harley and Smith (1956) and Harley and Jennings (1958) have demonstrated a probable sugar-accumulation process in lichen and in mycorrhizal tissues respectively. Studies on the general phenomenon of sugar uptake by higher plant tissues (Said 1941, 1950; Dormer and Street 1949; Said and Fawzy 1949; Said and Nada 1949; Street and Lowe 1950; Weatherley 1953, 1954, 1955; Porter and May 1955) have yielded only sketchy information on the specific process of sugar accumulation. Much of the necessary information is lacking because the contribution that respiration, starch synthesis, and particularly

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† The definitions of Kramer (1957) will be used, where “absorption” and “uptake” are general terms referring to the entrance of a substance into cells or tissues by any mechanism—diffusion, mass movement, or metabolic absorption; and where “accumulation” and “active transport” are terms describing an uptake which involves entrance to a cell and movement in a tissue against concentration gradients.



apparent free space (A.F.S.) uptake have made to the total sugar uptake has not been assessed. For example, the "active sugar uptake" process observed by Weatherley has since been shown (Pennell and Weatherley 1958) to be directed primarily towards polysaccharide synthesis rather than sugar accumulation *per se*. Even in the elegant experiments of Porter and May (1955) an unknown fraction of the radioactive sugars isolated from the leaf disks could have been unmetabolized molecules of the supplied sugar which had diffused into the A.F.S. In some cases (e.g. Said 1941, 1950) the amount of sugar taken up was such that, even assuming maximum possible A.F.S. absorption of the sugar, there was entry against a concentration gradient, indicating the operation of an active accumulation process.

There were reasons for expecting that sugar-cane tissues might be able to accumulate sugars. In extending studies on the respiration of sugar-cane (Bielecki 1958*a*) it was found that disks of sugar-cane tissue placed in aerated distilled water lost very little of their endogenous sugar to the water. Thus either the tonoplast is extremely impermeable to sugar movement or there is an accumulation mechanism in the cell which actively opposes the outward diffusional movement of sugar. The first is perhaps the simpler explanation, but raises the problem of explaining how the sugar originally became accumulated behind the impermeable tonoplast. Experiments were therefore carried out to examine the sugar-uptake process in sugar-cane tissue slices. An accumulation process is demonstrated, and some of its characteristics are established.

## II. MATERIALS AND METHODS

Sugar-cane is essentially a giant erect or semi-erect perennial grass with solid stems, in which the parenchyma surrounding the vascular bundles has become adapted to efficient sugar storage. This storage tissue was used in the following experiments. The cane samples were cut from a commercial crop, cv. Pindar, grown near Ingham, N. Qld., and taken by air-freight to Sydney. The cane was washed, placed in an open-ended polythene bag to reduce moisture loss, and stored at 5–8°C. Canes could be kept in this way for at least 3 weeks without any detectable changes in the behaviour of the tissue samples; in practice the maximum storage period was 14 days. Mature internodes at least 6 months old were taken from near the base of the cane. Experiments (described below) showed that the most suitable immature internodal material was given by the internode which had an internal sugar concentration of 3–6 per cent., in which intercalary growth was ceasing or had just ceased, and which subtended the oldest green leaf or youngest dying leaf.

The nodal tissue was removed from the selected internodes, and the outer shell of protective sclerenchyma split off with a thin sharp blade. The parenchyma core (which contains numerous vascular bundles) was split longitudinally into quarters, and transverse quadrant-shaped slices, 1.2–1.7 mm thick, were cut by hand with a razor-blade. Disks of tissue, 1.1 cm in diameter and 1.2–1.6 mm thick, prepared by slicing cores of tissue taken with a cork borer, were used in one experiment only, since their preparation resulted in unnecessary wastage of tissue, and could have caused excessive compression damage. Furthermore, because cane storage

tissues were dense (sp. gr. 1.05–1.10) neither disks nor slices could be made to circulate in the aerating solution in the normal way. The regularly shaped disks tended to clump, hindering aeration, while the irregular quadrant-shaped slices remained separate, allowing the surrounding solution to circulate through them. Slices were washed for 15–20 hr in 5–7 changes of distilled water, blotted dry, and weighed into samples, generally 60 g (240 slices).

The disk/solution ratio was 1 : 2–1 : 3 (w/v) and the concentration of sugar (sucrose or glucose) supplied in the external solution was less than half the concentration of that sugar in the tissue, and generally about one-twentieth. The amount of sugar taken up by the tissue was estimated by measuring the decrease in sugar concentration of the external solution. Where sucrose was supplied (except

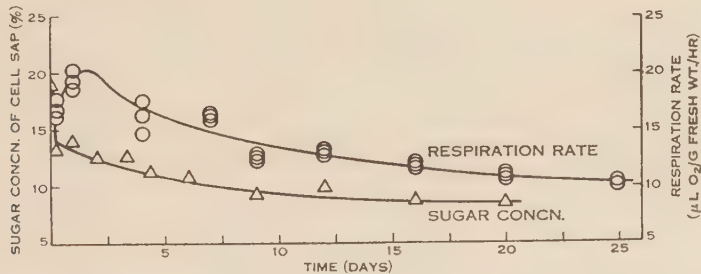


Fig. 1.—Change with time of respiration rate and sugar content of mature storage-tissue slices:  $\circ$  respiration rate ( $\mu\text{l O}_2/\text{g}$  fresh wt./hr);  $\triangle$  percentage sugar concentration of cell sap. Samples of 15 disks (1.55 g) used. Triplicate samples used for respiration measurements (individual values plotted). Single samples used for sugar-concentration measurements.

Table 2) the changes in percentage dry weight of the solution were measured, it being assumed that all changes were due to sucrose uptake. Where glucose was supplied (except Fig. 8) the changes in concentration of reducing sugars were measured, using a modified Somogyi sugar reagent method (Somogyi 1945). A slow outward leakage of sucrose from the tissue which occurred during the course of the experiment was estimated by the dry weight method, but not the Somogyi method, which determined changes in reducing sugar concentration only. The uptake rate as measured by the dry weight method was therefore 5–10 per cent. lower than that measured by the Somogyi method.

The sugar content of the tissue samples (Fig. 1; Table 1) was estimated by measuring the refractive index of the expressed sap with an Abbé refractometer. Because sucrose is the only major soluble component of sugar-cane sap, this gives a reliable measure of sugar content. Each flask plus its contents (including aerator) was weighed initially and reweighed during the course of the experiment to allow for any evaporation of water from the solution, though with humidification of the air supply this precaution was generally not necessary. Experiments were conducted at room temperature.

At pH 5.5, 0.07M phosphate buffer increased the respiration rate of the tissue slices 15-40 per cent., while at pH 7.0 and 8.0 the respiration was increased up to 90 per cent. This did not appear to be a normal salt-accumulation respiration, but resembled the "uncoupled" respiration obtained with dinitrophenol (Bielecki 1958*b*). To avoid possible complications, therefore, buffers were not used, though the tissue itself effectively buffered the solutions at pH  $5.4 \pm 0.3$ . No bactericide was used. Various methods were used to test for infection. The turbidity of the

TABLE 1

MOVEMENT OF SUGAR AGAINST A CONCENTRATION GRADIENT IN IMMATURE TISSUE SLICES

Expt. No.	Duration (hr)	Sugar Supplied	Final Concn. in External Solution (mg/ml)	Amount Absorbed in Second-stage Uptake (mg/g)	Ratio Absorbed/External Sugar Concn.	Endogenous Sugar Concn. (mg/g)	Ratio Tissue/External Sugar Concn.
XIII	100	Sucrose	10.76	23.79	2.21	52.7	7.12
			10.69	24.57	2.30		7.24
XV	30	Glucose	0.23	2.07	8.92	4.5-5.5	28-32
XVI	48	Sucrose	0.31	5.06	16.3	50-60	175-210
			0.26	5.33	20.5		210-250
XVII	43	Sucrose	1.02	3.04	2.98	62-67	64-70
		Glucose	0.87	3.36	3.76	9-11	14-17
		Fructose	0.45	4.18	10.75	7-9	25-30
XXIII*	24	Glucose	1.69	1.20	0.71		
			1.29	2.07	1.81		
			0.74	3.53	4.78		
			0.26	4.47	16.85		

\* Internodes of various maturities used.

solution was estimated; or solutions and slices were stained and examined microscopically; or the respiration rate of the sugar solution was measured at the end of the experiment. Under the conditions of vigorous aeration used, bacterial infection did not become significant with immature slices in the first 24 hr, or with mature tissues over a period of 100 hr. The mature tissues appeared to inhibit growth of bacteria and fungi.

### III. RESULTS

#### (a) *Respiration Drift and Sugar Loss in Mature Tissue Disks*

Mature tissue disks could be kept without infection or damage for periods of up to a month at 20°C, provided the distilled water was changed regularly. The pattern of respiration was determined by measuring the respiration rate of the

disks at intervals, using triplicate samples and standard Warburg manometry at 25°C (Fig. 1). The respiration rate fell steadily from 20 to 10  $\mu\text{l O}_2/\text{g}$  fresh wt./hr over the course of 550 hr. The respiration rate was thus of the same order as that of the single mature internodes (Bielecki 1958a).

The sugar concentration of sap extracted from the intact mature internodes was 19.0 per cent., but of sap extracted from disks 4 hr after cutting was 13.5 per cent. Two-thirds of the apparent dilution could be accounted for by the presence of water in the intercellular spaces (5 per cent. of the tissue volume as measured by vacuum injection) and in the volume of the cut cells (an estimated 17 per

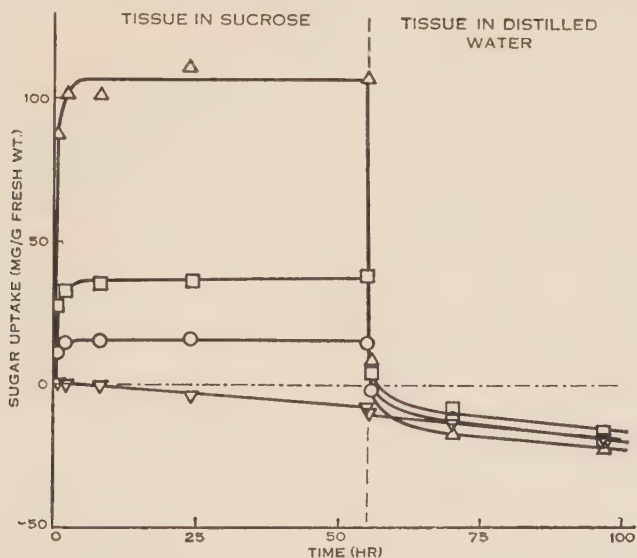


Fig. 2.—Sugar uptake by mature tissue slices from sucrose solutions of various concentrations, and its subsequent loss in distilled water:  $\nabla$  distilled water;  $\circ$  5 per cent. sucrose;  $\square$  10 per cent. sucrose;  $\triangle$  30 per cent. sucrose. At hour 55, tissue removed from sucrose solution, blotted, and placed in distilled water. Tissue sample weight 50.0 g; sucrose solution volume 150 ml; distilled water volume 150 ml.

cent. of the disk volume (Bielecki 1958b)). There may also have been some leakage from intact cells and some dilution by osmotic intake of water. Subsequent sugar losses were low, 0.2 per cent. per day (Fig. 1), respiration accounting for one-third and the remainder being lost either by leakage or occasional cell rupture.

#### (b) Sugar Uptake by Mature Tissue Slices

Slices of mature tissues from cane cut in July, with an internal sugar concentration of *c.* 10 per cent., rapidly took up sugars (sucrose and glucose), equilibrium being reached within the first hour, and the half-time of equilibration being 8 min. There was no further uptake of sugar over the next 54 hr. The amount of sugar taken up was exactly proportional to the sugar concentration of the



external solution. When the tissue was returned to distilled water, the absorbed sugar was rapidly and completely lost from the tissue (Fig. 2). Mannitol, generally regarded as being metabolically inert to most tissues, was taken up at exactly the same rate and to the same final equilibrium as sucrose (Fig. 3).

The effect of an anaerobic condition on the uptake of sugar by mature tissue slices was determined. The tissue sample was placed in a flask in distilled water, and aerated for 2 hr with oxygen-free nitrogen to remove any oxygen dissolved in the tissues. The flask was then inverted so that the water could be drawn off through the gas outlet, and the required sugar solution (pre-flushed with nitrogen)

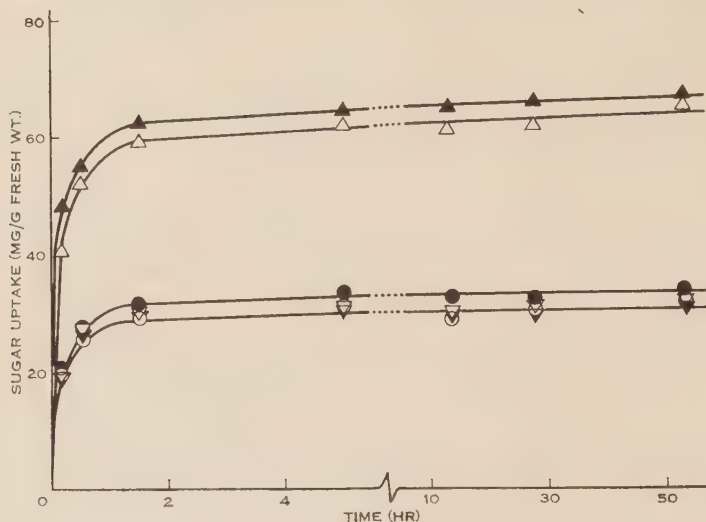


Fig. 3.—Sugar uptake by mature tissue slices in sucrose and mannitol under anaerobic conditions: ○ 10 per cent. sucrose, air; ● 10 per cent. sucrose, nitrogen; ▽ 10 per cent. mannitol, air; ▼ 10 per cent. mannitol, nitrogen; △ 20 per cent. sucrose, air; ▲ 20 per cent. sucrose, nitrogen. Tissue sample weight 60.0 g; solution volume 180 ml.

introduced without allowing any air to enter. The nitrogen flushing was then recommenced. The anaerobic conditions did not decrease the rate of sugar uptake or the amount absorbed at equilibrium (Fig. 3). These results all suggest that the sugar uptake observed was a passive uptake into the A.F.S. of the tissue.

### (c) Sugar Uptake by Immature Tissue Slices

Sugar uptake by immature tissue slices took place in two stages. In the first stage, there was a rapid initial uptake of sugar which ceased within an hour (half-time *c.* 8 min). The amount of sugar taken up in this stage was proportional to the concentration of the external solution (Fig. 4). This first stage in immature slices was identical to the complete uptake pattern in mature slices, and can be regarded as due to passive sugar movement into the A.F.S.

In the second stage there was a slow uptake of sugar which continued at a constant rate for at least 20 hr and up to 60 hr. The rate of uptake was independent of sugar concentration above 2 per cent. (Fig. 4). When dilute sugar solutions (0.25 per cent.) were supplied to the tissue, the amount of sugar taken up in this second stage (exclusive of A.F.S. sugar and endogenous sugar) was such that there must have been a 2- to 20-fold concentration of sugar in the tissue from the external solution (Table 1). The second stage of uptake can therefore be regarded as an accumulation of sugar into the osmotic volume of the tissue by a metabolic process.

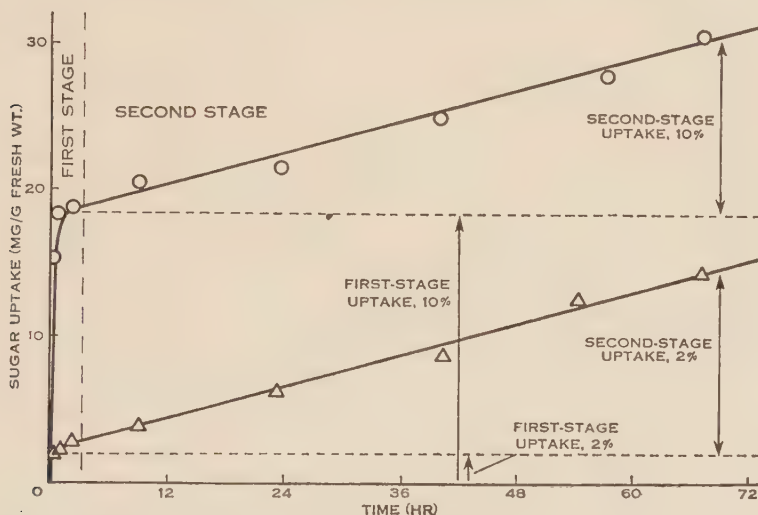


Fig. 4.—Sugar uptake by immature tissue slices from sucrose solutions of various concentrations:  $\Delta$  2 per cent. sucrose;  $\circ$  10 per cent. sucrose. Tissue sample weight 35 g; solution volume 125 ml. The first-stage (passive) uptake is proportional to the sugar concentration of the external solution, and the second-stage (accumulation) uptake is largely independent of concentration.

In solutions of mannitol the first stage of rapid uptake occurred, but the second stage of slow accumulation was practically absent (Fig. 5). When tissue slices which had previously been allowed to take up sugar from mannitol and sucrose solutions were blotted and returned to distilled water, sugar equivalent to the first-stage (A.F.S.) uptake was lost from the tissue to the water; while sugar equivalent to the second-stage (accumulation) uptake remained in the tissue (Fig. 5). The sucrose lost from the A.F.S. was then slowly taken into the tissue again by the continuing operation of the accumulation mechanism, at the original rate of accumulation, until the final equilibrium was established. At equilibrium, the endogenous sugar concentration was 8.1 per cent. (0.9 per cent. having entered the tissue by accumulation from the solution) and the solution concentration 0.017 per cent. The tissue which had been in mannitol re-accumulated the lost sugar only slowly. At least a part of the material which was re-accumulated may not

have been mannitol but sucrose and glucose, present in the solution through "leakage" of endogenous sugars from the tissue during the course of the experiment.

(d) *Effect of Anaerobiosis on Sugar Uptake in Immature Tissues*

When slices of immature tissue immersed in dilute sugar solutions were flushed with oxygen-free nitrogen instead of air, the rapid first-stage uptake was not affected, but the second-stage uptake was either greatly reduced or completely stopped (Figs. 6 and 7). Where the uptake was completely stopped, sugars already accumulated by the tissue leaked back into the solution (Fig. 7). When the accumulating sugar was glucose a considerable amount of sucrose appeared as the leaking

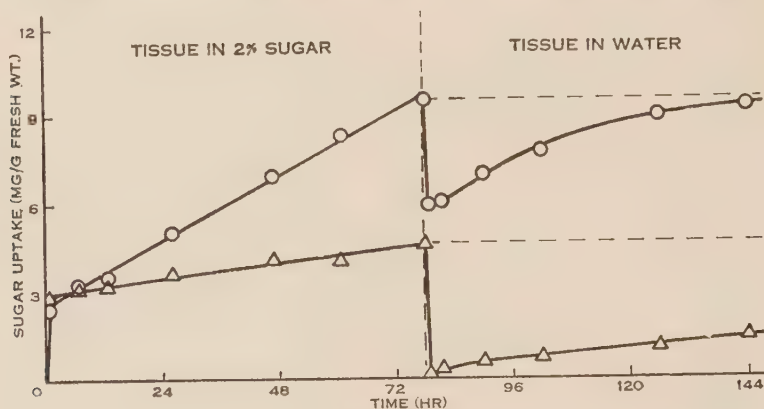


Fig. 5.—Uptake of sucrose and mannitol by immature tissue slices and their subsequent loss in distilled water: ○ 2 per cent. sucrose; △ 2 per cent. mannitol. Tissue sample weight 40.0 g; sugar solution volume 120 ml. At hour 78, tissue sample weight  $40.6 \pm 0.2$  g; water volume 120 ml. The almost complete loss of mannitol to the water indicates that most of this sugar was held in the A.F.S. The volume of the A.F.S. must have increased during the course of the experiment, probably through cells bursting. Only part of the sucrose taken up can have been in the A.F.S. since only 35 per cent. was lost when the tissue was placed in distilled water, and 85 per cent. of this was taken up again into the tissue.

sugar, and when sucrose was supplied, some hexose sugar also leaked from the cells. When the tissue was aerated in distilled water to remove any products of anaerobic respiration then returned to fresh sugar solution and aerated with air, sugar accumulation proceeded at the original rate. The inhibition of uptake and induction of leakage by anaerobiosis were therefore completely reversible (Fig. 7).

(e) *Comparative Uptake Rates of Various Sugars in Immature Tissues*

Comparative tests of accumulation were made on fructose, glucose, sucrose, glucose 1-phosphate, and mannitol. In 0.25 per cent. solutions, fructose was accumulated at a rate similar to or slightly higher than that of glucose. Glucose was accumulated at a rate generally 25 per cent. higher than that of sucrose on a weight basis, and more than double on a molar basis (Fig. 8). However, when glucose and sucrose were supplied to the tissue together, glucose accumulation appeared to be

largely suppressed at the expense of sucrose accumulation (Table 2). The total uptake of the two sugars was more than that of either singly on a weight basis, but the same as the uptake of glucose alone on a molar basis.

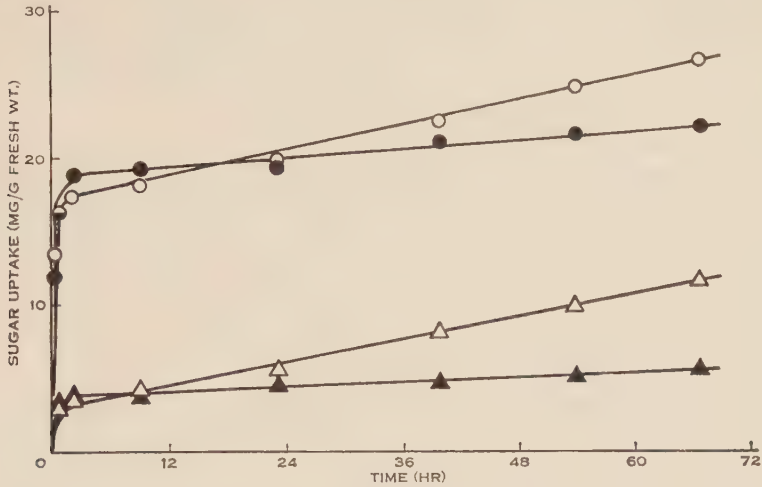


Fig. 6.—Effect of anaerobic conditions on sucrose uptake by immature tissue slices: △ 2 per cent. sucrose, air; ▲ 2 per cent. sucrose, nitrogen; ○ 10 per cent. sucrose, air; ● 10 per cent. sucrose, nitrogen. Tissue sample weight 25.0 g; solution volume 75 ml.

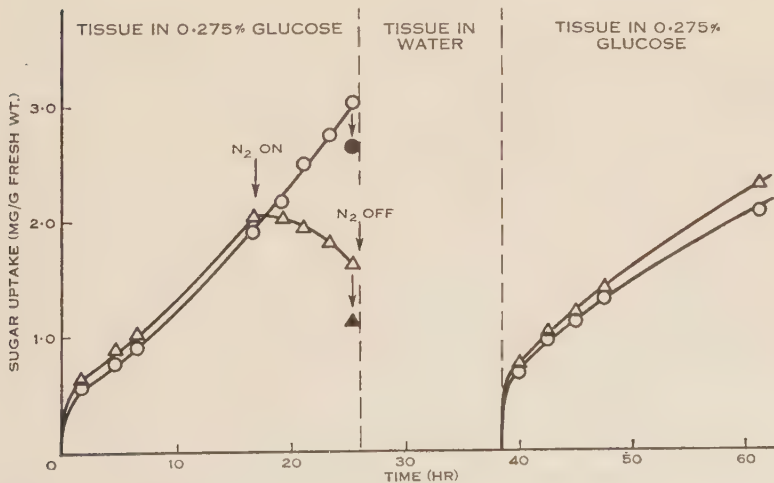


Fig. 7.—Reversible nature of the effect of anaerobic conditions on glucose uptake by immature tissue slices: ○ 0.275 per cent. glucose, aerated with air; △ 0.275 per cent. glucose, aerated with air till hour 17, then with nitrogen till hour 26, thereafter with air. Both samples placed in distilled water at hour 26 and washed till hour 38, then returned to 0.275 per cent. glucose solution. Tissue sample weight 110 g; glucose solution volume 240 ml; wash volume four changes of 350 ml. —●— Sucrose leakage from tissue, control. —▲— Sucrose leakage from tissue, anaerobic sample.



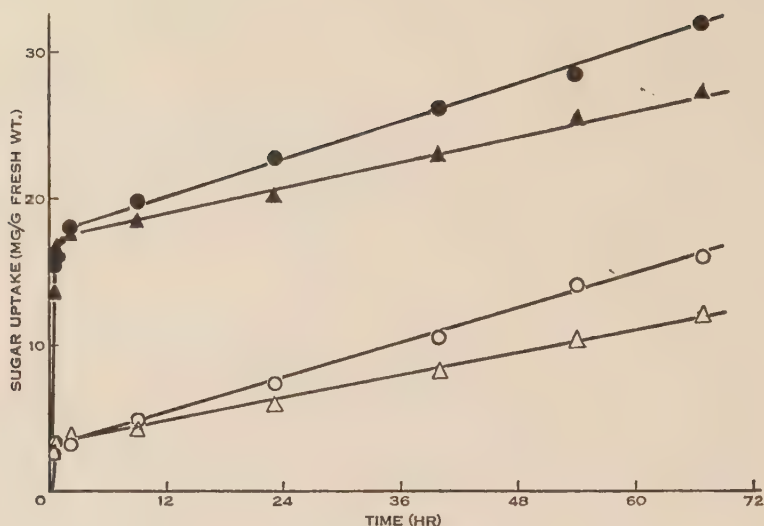


Fig. 8.—Comparative rates of uptake of sucrose and glucose by immature tissue slices:  $\triangle$  2.0 per cent. sucrose;  $\circ$  2.0 per cent. glucose;  $\blacktriangle$  10.0 per cent. sucrose;  $\bullet$  10.0 per cent. glucose. Tissue sample weight 25.0 g; solution volume 75 ml.

TABLE 2

RATE OF ACCUMULATION OF SUCROSE AND GLUCOSE FROM SIMPLE AND MIXED SOLUTIONS  
Sugar concentrations given as mg/g tissue/hr. Values in parenthesis are these concentrations expressed as  $\mu\text{M/g}$  tissue/hr

		Solution Supplied to Tissue		
		Glucose (2.5 mg/ml)	Sucrose (2.5 mg/ml)	Glucose plus Sucrose (each 2.5 mg/ml)
Glucose	Uptake	+0.200 (1.11)		
	Leakage†		−0.018 (0.10)	+0.078 (0.43)
Sucrose*	Uptake		+0.178 (0.53)	+0.194 (0.56)
	Leakage	−0.020 (0.06)		
Net sugar uptake		+0.180 (1.05)	+0.160 (0.43)	+0.272 (0.99)

\* Changes in glucose concentration measured by changes in reducing sugars; net sugar uptake measured by changes in total (reducing plus non-reducing) sugars; and changes in sucrose concentration estimated by difference. Tissue sample weight 80 g, solution volume 160 ml.

† Part or all of this sugar could have come from hydrolysis of sucrose in the solution; but results of other experiments indicate that most came from leakage.

These results indicated that glucose might not be accumulated as such. The possibility was considered that sugar uptake might occur through glucose 1-phosphate. Glucose 1-phosphate was not accumulated under normal experimental conditions (Fig. 9). No significant hydrolysis occurred during the experiment. Phosphate ion by itself ( $2 \times 10^{-3}M$ , pH 5.5) did not increase the uptake of glucose. Sugar-cane tissues showed little tendency to accumulate mannitol.

(f) *Fate of Accumulated Sugars*

Starch formation could account for part or all of the sugar taken up. Immature tissue slices were tested for starch, before and after a period of accumulation, by perchloric acid extraction and an iodine colour test. No trace of starch

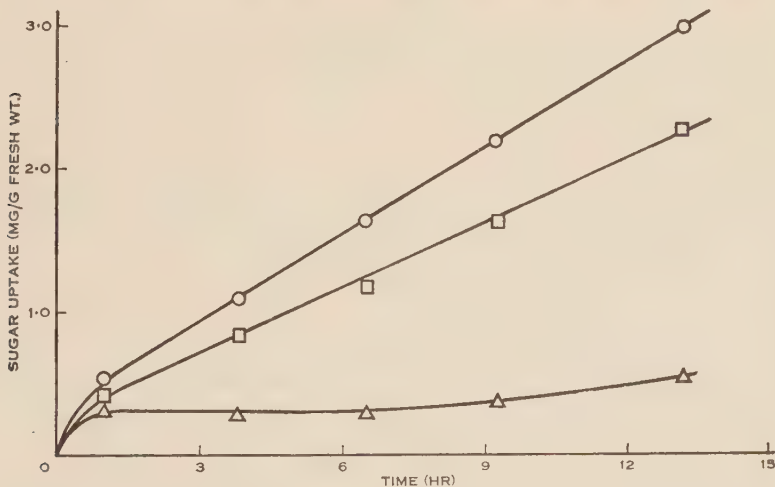


Fig. 9.—Comparative rates of uptake of sucrose, glucose, and glucose 1-phosphate by immature tissue slices:  $\square$  0.225 per cent. (6.3 mM) sucrose;  $\circ$  0.225 per cent. (12.5 mM) glucose;  $\triangle$  0.326 per cent. (12.5 mM) potassium glucose 1-phosphate. Tissue sample weight 80 g; solution volume 160 ml.

could be demonstrated in either sample. Microscopic inspection of stained sections also failed to reveal any starch, though sugar accumulation equivalent to 1 per cent. of the fresh weight had occurred.

In many tissues, addition of sugars causes an immediate rise in the respiration rate as the substrate is utilized. Part or all of the sugar taken up by sugar-cane could have been used in this way. Also, accumulative processes require energy to transport materials against a gradient; and therefore an increase in respiration similar to the "salt respiration" increase (Robertson and Wilkins 1948) would be expected to follow addition of sugars to immature sugar-cane tissue slices. Respiration rates of such slices were measured by conventional Warburg manometry before and after addition of glucose, sucrose, and mannitol, and compared with the accumulation rate of the sugar (Table 3). Accumulation of sugar was found to be associated with a 30–40 per cent. increase in respiration. The loss of sugar

in increased respiration accounted for 12 per cent. of the sugar taken up in the tissue.

An attempt was made to find whether the sugar disappearing from the external solution could be recovered as sugar from the tissue slices. Three paired samples of tissue were used: (i) tissue aerated for 103 hr in 2 per cent. sucrose, then washed in four changes of distilled water over 3 hr to remove the freely diffusible sugar held in the A.F.S.; (ii) tissue aerated for 49 hr in distilled water, then for 54 hr in 2 per cent. sucrose, then washed as above; (iii) tissue aerated for 100 hr in distilled water, then for 3 hr in 2 per cent. sucrose, then washed as above. The sucrose uptake was followed by measuring the dry weight changes in the external solution. At the end of the experiment, after the tissue samples had been washed

TABLE 3  
COMPARISON OF "ACCUMULATION RESPIRATION" RATE AND SUGAR ACCUMULATION RATE

	Sugar Supplied to Tissue		
	Sucrose	Glucose	Mannitol
Initial respiration rate ( $\mu\text{l O}_2/\text{g/hr}$ )	46.0	48.5	48.0
Respiration rate in sugar ( $\mu\text{l O}_2/\text{g/hr}$ )	60.0	68.0	51.5
Respiration increase ( $\mu\text{l O}_2/\text{g/hr}$ )	14.0	19.5	3.5
Respiration increase as sugar loss ( $\mu\text{g/g/hr}$ )	17.8	26.2	4.4*
Sugar uptake ( $\mu\text{g/g/hr}$ )	148.0	189.0	20.8

\* Assuming mannitol respired.

in distilled water, each sample was homogenized in a blender with 70 per cent. ethanol, the slurry filtered, and the residue extracted twice more with 70 per cent. ethanol. This procedure left less than 0.3 per cent. of the sugar in the tissue. Aliquots of the tissue extracts were analysed for sugar content by the Somogyi method, and the increase in sugar content through each period of accumulation was calculated by difference (Table 4). Less sugar appeared in the tissue than was lost from the solution. The 8 per cent. discrepancy probably represents the amount of sugar used in maintaining the accumulation respiration.

#### (g) *Effect of Tissue Maturity on Sugar Uptake*

The cane variety Pindar is a vegetatively propagated clone, and under favourable conditions crop growth may be so regular that it is possible to select uniform samples of internodes of various maturities. Eight closely matched cane tops were selected and four samples of tissue were cut from each cane top. In sugar-uptake

experiments it was found that the older the tissue the greater the first-stage uptake and the less the rate of uptake in the second stage (Table 5).

TABLE 4

COMPARISON OF SUCROSE UPTAKE AS MEASURED BY LOSS IN DRY WEIGHT OF EXTERNAL SOLUTION AND INCREASE IN TISSUE SUGAR CONCENTRATION

Absorption Period* (hr)	Sugar Uptake as Loss from External Solution*		Sugar Uptake as Appearance in Tissue		
			Reducing Sugar (mg/g)	Non-reducing Sugar (mg/g)	Non-reducing Sugar (mg/g/hr)
51	11.9	0.234	0.1	11.4	0.224
	11.4	0.224	0.5	11.0	0.216
100	23.8	0.238	0.0	23.3	0.233
	24.6	0.246	0.0	22.0	0.220

\* For sugar appearance in tissue, absorption period derived from (ii) minus (iii) and from (i) minus (iii)—see text, p. 214. For sugar loss from external solution "zero time" taken 3 hr after sugar solution added to (ii) and (i); thus making time intervals the same for the two methods and avoiding making uptake measurements until after diffusional, A.F.S. uptake complete.

At certain times of the year, even mature tissues could be shown to accumulate sugars. In March (the middle of the growing season) mature internodes (over 6 months old, from the base of the stem) had a low endogenous sugar content,

TABLE 5

INTERNODE AGE AND SIZE AS RELATED TO RATE OF SUGAR ACCUMULATION

Group	Phase of Growth of Internode	Mean Weight (g)	Mean Length* (cm)	A.F.S. (%)	Rate of Sugar Accumulation (mg/g/hr)
1	At final width, elongation starting	16.0 ± 2.4†	3.00 ± 0.43†	10	0.270
2	Elongation active	22.1 ± 2.9	4.30 ± 0.44	10	0.174
3	Elongation finishing	34.0 ± 3.9	6.61 ± 0.67	15	0.116
4	Elongation complete	47.1 ± 3.5	8.85 ± 0.44	19	0.084

\* The internodes used were smaller than is usual at this stage of development.

† Standard deviation.

c. 10 per cent. Tissue slices from these internodes accumulated sucrose from a 0.5 per cent. solution at a rate one-third that of the immature tissue (Table 6).



## IV. DISCUSSION

In mature sugar-cane tissue slices, the sugar uptake had the characteristics of a non-metabolic (diffusional) uptake into the A.F.S. (Briggs and Robertson 1957). Sugar uptake reached an equilibrium within an hour, the equilibrium was closely proportional to the concentration of the sugar solution supplied, independent of the nature of the sugar, and unaffected by anaerobic conditions: the sugar taken up was completely and rapidly released from the tissue when the tissue was placed in distilled water. From the amount of sugar taken up at equilibrium, the percentage A.F.S. of the tissue could be calculated, and was found to be 15–21 per cent. This is of the same order as has been found for other plant tissues. The volume of the A.F.S. appeared to be greatest in the tissues with the highest proportion of cell wall material. This ranged from 11 to 17 per cent. of the tissue

TABLE 6  
ACCUMULATION RATE IN TISSUE SLICES AS RELATED TO INTERNODE AGE AND MATURITY

Internode Maturity	Month	Sugar Content (%)	Storage in the Field	Accumulation Rate <i>in vitro</i> (mg/g/day)
Immature	December	4	Active	4.30
Immature	March	4	Active	4.75
Immature	July	5	Active	4.15
Maturing	July	8	Medium	2.95
Mature	July	16	Little or none	<0.30
Mature	March	10	Slow	2.10

fresh weight, as estimated by tissue fragmentation and dry weight analyses. The vascular bundles formed about 7 per cent. of the tissue volume: part of this tissue was non-living, and would have contributed to the A.F.S. Cut cell surfaces and intercellular spaces, 3–6 per cent. of the tissue volume as estimated by water injection, were sufficient to account for the remainder of the A.F.S. volume. The cytoplasm was too thin to be distinguished microscopically from the cell wall in an unfixed section, so the volume of the cytoplasm was probably less than 3 per cent. of the cell volume, and too small to determine whether or not it formed part of the A.F.S.

In the immature tissue slices, the first stage of uptake also showed all the characteristics of an A.F.S. uptake: the uptake was rapid, independent of the nature of the sugar and proportional to the sugar concentration, and unaffected by anaerobic conditions; and when the tissue was returned to distilled water, sugar equivalent in amount to the first-stage uptake was rapidly released from the tissue. From the amount of sugar taken up in the first stage, the percentage A.F.S. of this tissue was found to be 10–18 per cent.

The second stage of sugar uptake in immature tissue slices had different characteristics: the uptake was slow, continued for a long time, was dependent

on the nature of the sugar but more or less independent of its concentration, and was affected markedly by anaerobic conditions; the sugar taken up in this stage was not released when the tissue was placed in distilled water. The amount of sugar taken up in this stage was such that the sugar from the external solution was increased in concentration 2–20 times in passing into the tissue. If the endogenous sugar concentration of the tissue, 3–6 per cent., is taken into account, it can be shown that sugar movement occurred against a 10- to 200-fold gradient (Table 1). The second-stage uptake therefore had the characteristics of an active accumulative process. The two-stage uptake pattern of sugars in sugar-cane is closely analogous to that observed for salt (ion) uptake in other plant tissues (Hope and Stevens 1952; Epstein 1955; Kramer 1957).

Since the sugar taken up in the second stage could not be recovered by rinsing the tissue in water, it must either have been transformed into insoluble compounds, or accumulated behind a permeability barrier. Starch did not occur, there was no sign that any cell multiplication or cell expansion had taken place, and most of the sugar taken up could be recovered as such by alcohol extraction of the tissue. The observed uptake of sugar must therefore have occurred through a true accumulation process.

One characteristic of accumulation processes is their frequent discrimination between closely related molecular structures. Opinion is divided as to whether sucrose is taken up as such into plant tissues or whether it must first be hydrolysed by the tissue. Since it is often the cell surfaces exposed by cutting that are responsible for the hydrolysis (Hassid 1958) the apparent dependence of uptake on hydrolysis may sometimes be an artifact (see Harley and Smith 1956). Though sucrose was rapidly accumulated by sugar-cane tissue there was negligible hydrolysis in the external solution, the traces of hexose which occurred apparently coming by slow diffusion from the cell vacuoles or by occasional rupture of cells. Some support to the theory that sucrose was taken up as such is given by the observation that when tissue was allowed to accumulate in sucrose, the sucrose content of the tissue increased while hexose remained constant. The rate of sugar accumulation from a solution of 0.25 per cent. glucose + 0.25 per cent. sucrose was 1.5 times as great as from a solution of 0.25 per cent. glucose alone (Table 2); this also suggests that sucrose is absorbed as such.

The results of these experiments do not provide much information on the mechanism of the sugar-accumulation process. As has been suggested for amino acid accumulation (Birt and Hird 1956), sugar accumulation could occur as a type of salt-accumulation process. If this were so, sucrose (or glucose) would presumably be converted to an ionic form such as the sucrosyl phosphate ion. This would require energy in the form of energy-rich phosphate bonds ( $\sim$ P). Further energy would be required to provide for the accumulation of this ion against a concentration gradient. If it is assumed that the respiration increment which occurred when sucrose and glucose were added to the slices provided the energy for the accumulation of those sugars, then 17.8  $\mu$ g sucrose was respired to provide for the accumulation of 148.0  $\mu$ g sucrose, and 26.2  $\mu$ g glucose was respired to provide for the accumulation of 189.0  $\mu$ g glucose (from Table 3). The energy yield

of aerobic respiration is approximately 38  $\sim$ P per hexose molecule completely respired. Hence, 9  $\sim$ P were generated per sucrose molecule accumulated, and 5  $\sim$ P per glucose molecule accumulated. If glucose was first converted to sucrose before being accumulated (as suggested by data in Figure 9 and Table 2), requiring 1  $\sim$ P per sucrose molecule (Shukla and Prabhu 1959), there would again be 9  $\sim$ P available per sucrose molecule. This, for example, would be sufficient to permit phosphorylation of the sucrose molecule (requiring 1–2  $\sim$ P) and still provide for its subsequent accumulation at an efficiency of about 20 per cent. assuming the accumulation mechanism proposed by Lundegårdh (1945) and Robertson and Wilkins (1948).

In the field, during active photosynthesis, the sugar concentration of the leaf is less than 2–3 per cent. of the fresh weight, 70 per cent. of the sugar being sucrose (Hartt 1935); but in the young and immature internodes the concentration is 4–10 per cent. (75–85 per cent. as sucrose), and in the old mature internodes at the base of the stem the concentration may be as high as 20 per cent. (95 per cent. as sucrose) (Lal and Srivastava 1945; Hes 1949). In the mature internodes the sugar concentration is lowest (c. 12 per cent.) in the season when cane growth is at a maximum, and rises to a peak in the “ripening” season (Das 1936); these two periods being in summer and in late autumn–early winter respectively in Australia (Lowndes 1956). At least a part of the sugar stored in the mature internodes during ripening comes directly from current photosynthesis in the leaves (Hartt and Burr (1953). *In vitro*, the sugar accumulation rate shown by a particular tissue slice sample was closely related to the metabolic state of the parent tissue in the field. The maximum accumulation rate, c. 6.0 mg/g tissue/day, occurred in slices of tissue taken from internodes in which cell division and enlargement had just ceased, and which were starting to store sugars rapidly as part of the maturation process. At this time in the field, the endogenous sugar concentration rises from 3 to 11 per cent. in 2–4 weeks (2.8–5.6 mg/g tissue/day). In older internodes where the storage was slower, the rate of sugar accumulation in tissue slices was correspondingly less (Table 6). In fully mature tissues (sugar concentration of tissue over 16 per cent., June–August), there was no detectable accumulation. The decreased uptake was not a function of the increased concentration gradient between solution and tissue, since increasing the solution concentration to 25 per cent. did not increase the rate of uptake. Thus the laboratory behaviour of the tissue slices is closely comparable to the field behaviour of the intact tissue, suggesting that it might be possible to use laboratory experiments to screen new cane varieties for their ability to store sugar.

#### V. ACKNOWLEDGMENTS

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# THE PHYSIOLOGY OF SUGAR-CANE

## IV. EFFECTS OF INHIBITORS ON SUGAR ACCUMULATION IN STORAGE TISSUE SLICES

By R. L. BIELESKI\*

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### *Summary*

Various metabolic inhibitors, at pH 5.5, affected sugar accumulation in immature sugar-cane storage tissues. The rate of accumulation was reduced by  $10^{-5}\text{M}$  mercuric ion,  $10^{-4}\text{M}$  *p*-chloromercuribenzoate, cyanide, and cupric ion, and  $2 \times 10^{-3}\text{M}$  phloridzin. Accumulation was completely inhibited and sugar leakage induced by  $10^{-5}\text{M}$  dinitrophenol,  $10^{-4}\text{M}$  mercuric ion, and  $10^{-3}\text{M}$  *p*-chloromercuribenzoate, cyanide, cupric ion, azide, arsenate, and iodoacetate. The effects of  $10^{-5}\text{M}$  dinitrophenol and  $10^{-4}\text{M}$  cyanide were reversible, but that of  $10^{-3}\text{M}$  cyanide was irreversible. Only slight effects were produced by borate, phosphate, and magnesium ion.

The behaviour of sugar-cane sugar accumulation towards inhibitors was similar to that of other transport mechanisms, being somewhat more sensitive than most plant processes and less so than animal ones. It differed from sugar accumulation in animal tissues in showing no specific sensitivity to phloridzin. The results suggest that sugars are contained within the immature storage cell by the continuous operation of a metabolic storage mechanism rather than by an impenetrable barrier to sugar diffusion.

### I. INTRODUCTION

It has been shown that sugars can be taken up against a concentration gradient into sugar-cane storage tissues through the operation of an active accumulation mechanism (Bieleski 1960). This was found to have features in common with other accumulation mechanisms (Brown and Danielli 1954; Robertson 1956) and appeared closely allied to various active phloem translocation processes (Esau, Currier, and Cheadle 1957). Several metabolic inhibitors have been shown to inhibit these processes. The most widely used inhibitor has been 2,4-dinitrophenol (DNP),  $10^{-5}$ – $10^{-3}\text{M}$ , which has been found by various authors to inhibit phosphate and sugar transport in the phloem; sugar uptake in yeast, in bacteria, and in animal cells; salt accumulation in storage tissues, in algal tissues, and in yeast cells; amino acid accumulation; auxin uptake and translocation; and streptomycin uptake by *Nitella* cells. Partially or completely anaerobic conditions have also been shown to affect a wide variety of such processes. The other inhibitors used fall into two main groups: respiration inhibitors, such as cyanide and carbon monoxide; and sulphhydryl (–SH) group inhibitors, such as cupric ion, mercuric ion, *p*-chloromercuribenzoate (PCMB), and iodoacetate (James 1953*a*; 1953*b*). A considerable body of evidence indicates that the glycoside phloridzin (phlorrhizin, phlorizin, phlorrhidzin),  $10^{-6}$ – $10^{-5}\text{M}$ , can act as a specific inhibitor of sugar uptake in animal

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cells (LeFevre 1948; Wilbrandt 1954; Crane, Field, and Cori 1957; Riklis and Quastel 1958*b*); though in higher concentrations it also appears to act as an inhibitor of phosphate transfer (James 1953*a*).

Despite the wide variety of accumulation and translocation systems which have been studied, a consistent pattern of behaviour to the various inhibitors has been found. Transport was largely or completely inhibited by  $10^{-4}$ M DNP, and in some cases there was an outward "leakage" of solutes. Anaerobic conditions,  $10^{-3}$ M potassium cyanide, and (in plant tissues) 95 per cent. carbon monoxide in the dark all caused a partial or complete inhibition of active transport. Most of the processes

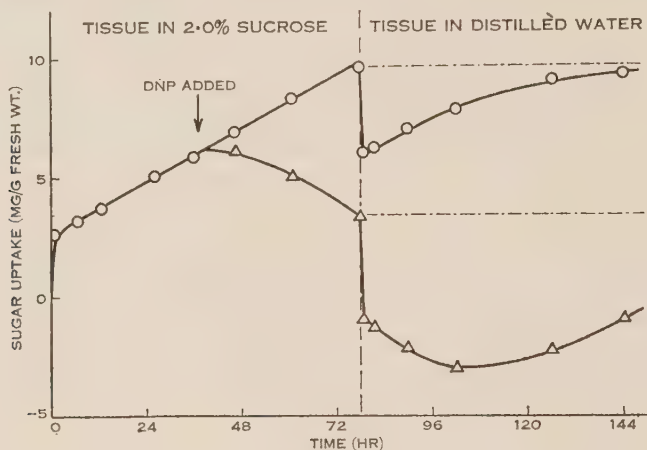


Fig. 1.—Inhibition of sugar accumulation in immature tissues by DNP, and its reversal by washing: ○ 2 per cent. sucrose; Δ 2 per cent. sucrose, made to  $10^{-5}$ M DNP by addition of DNP at hour 37. Both samples placed in distilled water at hour 78. Tissue sample weight 40 g; solution volume 120 ml; water volume 120 ml. Treatments in duplicate; mean values plotted, individual values agreeing to within  $\pm 0.2$  mg/g fresh weight.

were found to be extremely sensitive to -SH inhibitors. Phloridzin in concentrations as low as  $10^{-6}$ M was an effective inhibitor of sugar accumulation in animal tissues of various kinds. There is little information on the effect of phloridzin on plant tissues.

Only a few attempts have been made to increase accumulation and translocation rates by adding various substances to the medium. Borate has been claimed to increase the rate of translocation of sugars in soybean (see Dugger, Humphreys, and Calhoun 1957), potassium ion to stimulate glucose uptake in animal intestines (Riklis and Quastel 1958*a*, 1958*b*), and calcium, magnesium, and manganese ions to stimulate sugar uptake in yeast cells (Rothstein 1954).

This paper describes the effects of a range of inhibitors on the process of sugar accumulation in the storage tissues of sugar-cane. It conformed to the general pattern of behaviour of active transport systems towards inhibitors. Various compounds were tested for their ability to increase the rate of sugar uptake.

## II. MATERIALS AND METHODS

The sugar-cane used, cv. Pindar, was cut from a commercial crop grown at Ingham, N. Qld., and sent by air-freight to Sydney. Mature and immature internodes were selected as previously described (Bieleski 1960) and slices of tissue 1.2–1.7 mm thick cut from the storage parenchyma as before. The slices were washed in four to six changes of distilled water over 15–20 hr, then blotted dry. Weighed samples, generally 60 g (240–300 slices), were placed in aerated sugar solutions, usually 0.25 per cent. glucose, and the rate of sugar accumulation or leakage was measured in terms of changes in sugar concentration of the external solution.

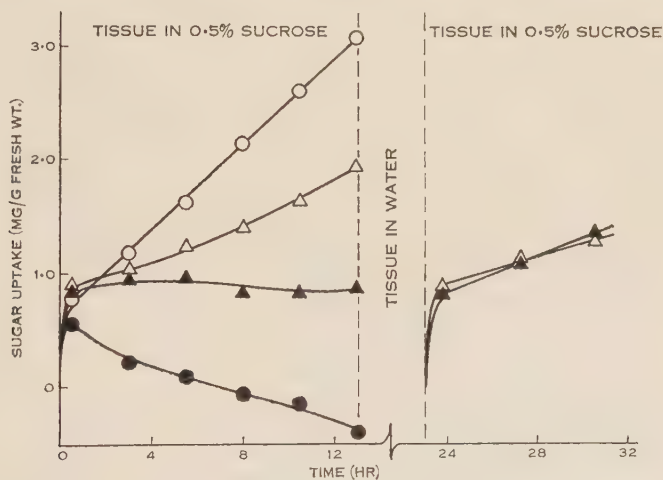


Fig. 2.—Inhibition of sugar accumulation in mature tissues by DNP, and its reversal by washing:  $\Delta$  mature tissue in 0.5 per cent. sucrose;  $\blacktriangle$  mature tissue in 0.5 per cent. sucrose +  $10^{-5}$ M DNP;  $\circ$  immature tissue in 0.5 per cent. sucrose;  $\bullet$  immature tissue in 0.5 per cent. sucrose +  $10^{-5}$ M DNP. At hour 13 mature tissue samples removed from sugar solutions, washed in four changes of distilled water over 10 hr, then replaced in fresh 0.5 per cent. sucrose solution without DNP. Immature samples discarded at hour 23 since they showed signs of damage or infection. Tissue sample weight 70 g; solution volume 140 ml, water volume 200 ml. Duplicate samples used in all treatments; mean values plotted, individual values agreeing to within  $\pm 0.2$  mg/g fresh weight.

Aliquots of the external solution were taken at various times, and sugars estimated by the method of Somogyi (1945). The effect of a compound was determined either by comparing the rates of sugar accumulation in a single sample before and after adding the inhibitor (e.g. Fig. 1) or by comparing the accumulation rates in a control sample and a sample to which the compound to be tested had been added (e.g. Fig. 2). Buffers were unnecessary and undesirable (Bieleski 1960) as the tissue kept the solution at pH 5.2–5.7 throughout all experiments. Inhibitor solutions were adjusted to pH 5.5 before use. Where particularly volatile inhibitors such as cyanide were used, closed-circuit aeration was employed to avoid loss of the inhibitor. The volume of air included in the circuit was such that the tissue respiration during the experiment did not lower the oxygen concentration of the air



below 0.190 atm (oxygen concentration of air 0.205 atm at start of experiment), while at the same time the inhibitor concentration in solution was substantially unaltered.

### III. RESULTS

#### (a) *Inhibition by Dinitrophenol*

Sugar accumulation in mature and immature tissues was completely inhibited by  $10^{-5}$ M DNP; and endogenous sugars were slowly lost from the tissue to the external solution (Figs. 1 and 2). When the inhibited tissue was placed in distilled water, the sugar leakage continued for about 30 hr, but eventually the tissue

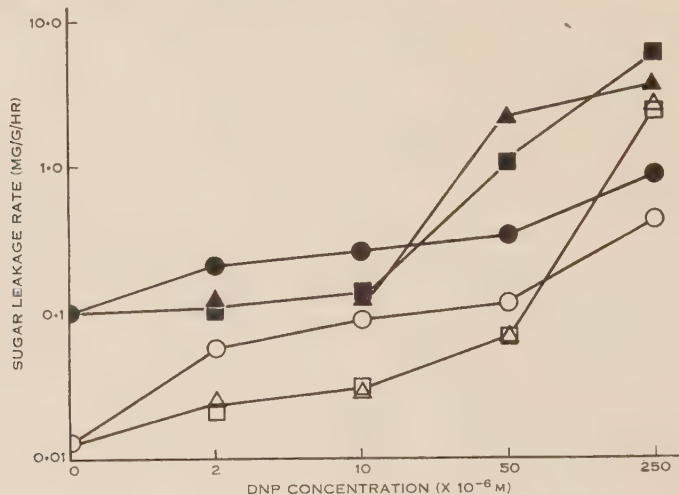


Fig. 3.—Effect of concentration of DNP on sugar leakage rate from mature and immature tissues: ● mature tissue, hour 0–5; ■ mature tissue, hour 5–12; ▲ mature tissue, hour 12–23; ○ immature tissue, hour 0–5; □ immature tissue, hour 5–12; △ immature tissue, hour 12–23. Tissue sample weight 55 g; solution volume 165 ml. Duplicate samples used; mean values plotted, individual values agreeing to within  $\pm 6$  per cent. of mean value.

recovered from the inhibition and commenced to re-accumulate the sugar which had been lost to solution (Fig. 1). When the inhibited tissue was washed in several changes of distilled water and then replaced in fresh dilute sugar solution, the recovery was more rapid and complete (Fig. 2).

It appeared possible that the loss of sugar from the tissue during DNP inhibition might occur because a normal slow diffusion of sugar through the tonoplast (under the influence of the high concentration gradient) was no longer being opposed by the energy-requiring accumulation mechanism. If this were so, the rate of sugar leakage from mature and immature tissues under various conditions might yield some information on the relative permeability of their cell tonoplasts. Although in any one experiment the replicates agreed well on the relative effects of DNP on mature and immature tissues, results varied from one experiment to another. The following example illustrates some of the factors involved.

The effect of a range of DNP concentrations on the rate of sugar leakage from mature and immature tissue slices was studied (Fig. 3). Over the first 5 hr the two types of tissue were affected in the same way by the DNP. The rate of leakage was several times greater from the mature tissue, and in both tissues it was about 10 times greater in  $2.5 \times 10^{-4}M$  DNP than in distilled water. Compared with the first 5 hr, the rates of leakage during the next 7 hr and during the following 11 hr were lower for the lower DNP concentrations, and higher for the higher

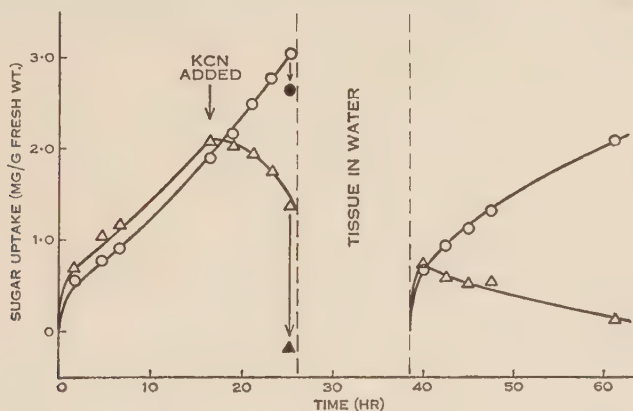


Fig. 4.—Effect of cyanide on sugar accumulation in immature tissues: ○ 0.275 per cent. glucose; △ 0.275 per cent. glucose, made to  $10^{-3}M$  cyanide by addition of cyanide at hour 17. At hour 26, both samples removed from sugar solutions, washed in four changes of distilled water over 12 hr, then replaced in fresh 0.275 per cent. glucose without cyanide. Tissue sample weight 110 g; solution volume 240 ml; water volume 350 ml. —●— Sucrose leakage from control tissue. —▲— Sucrose leakage from cyanide-treated tissue.

concentrations. Sugar leakage became very rapid from mature slices in solutions above  $1 \times 10^{-5}M$  DNP, and from immature slices in solutions above  $5 \times 10^{-5}M$  DNP. The most dilute DNP solutions, particularly when bathing the mature tissue, showed a definite tendency to lose their characteristic yellow colour, indicating that the tissue may have been inactivating or absorbing the DNP.

#### (b) Inhibition by Cyanide, Azide, and Arsenate

Potassium cyanide,  $10^{-3}M$ , completely inhibited sugar accumulation and induced a rapid leakage of sugar from the tissue (Fig. 4). Though glucose was the only sugar supplied in the solution originally, 74 per cent. of the sugar leaking out from the tissue was non-reducing sugar (presumably sucrose) and only 26 per cent. was reducing sugar. Thus the leaking sugar may have come mainly from the endogenous sugar in the cell vacuole, the accumulated sugar may have been converted to sucrose during its accumulation, or more probably both factors may have been involved. In the control sample, a small amount of non-reducing sugar also appeared in the external solution over the course of the experiment, indicating that there

was a small outward movement of sugar from the tissue even while there was a net accumulation of total sugar. When the tissue was washed in several changes of distilled water then returned to fresh dilute sugar solution, there was no accumulation, and leakage continued at a reduced rate. Hence, unlike anaerobic inhibition (Bielecki 1960) and  $10^{-5}\text{M}$  DNP inhibition, the  $10^{-3}\text{M}$  cyanide inhibition was not readily reversible. In a  $10^{-4}\text{M}$  solution, cyanide also completely inhibited sugar accumulation, and induced a slight sugar leakage which ceased after 3 hr. When the tissue was washed in several changes of distilled water then returned to fresh sugar solution, the accumulation recommenced at approximately the original uninhibited rate (Fig. 5).

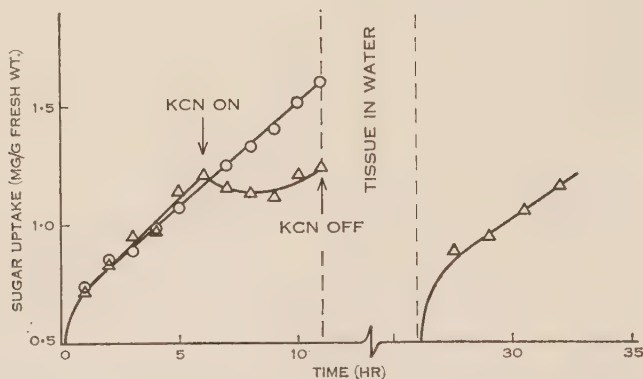


Fig. 5.—Effect of cyanide on sugar accumulation in immature tissues: ○ 0.275 per cent. glucose; △ 0.275 per cent. glucose made to  $10^{-4}\text{M}$  cyanide by addition of cyanide at hour 5.5. At hour 11, both samples removed from sugar solutions, washed in four changes of distilled water over 15 hr, then replaced in fresh 0.275 per cent. glucose without cyanide. Tissue sample weight 150 g; solution volume 300 ml; water volume 350 ml.

Sodium azide and sodium arsenate, each at a final concentration of  $10^{-3}\text{M}$ , both completely inhibited sugar accumulation and induced rapid sugar leakage from the tissue (Table 1).

### (c) Inhibition by Sulphydryl Group Inhibitors

Sodium iodoacetate,  $10^{-3}\text{M}$ , completely inhibited sugar accumulation and caused rapid sugar leakage (Table 1). Mercuric ion (mercuric chloride) caused no inhibition of sugar accumulation in sugar-cane tissues when at  $10^{-6}\text{M}$ , a concentration which is known to affect sugar accumulation in animal tissues. At  $10^{-5}\text{M}$ , mercuric ion caused a slight (5–10 per cent.) reduction in the rate of sugar accumulation; while at  $10^{-4}\text{M}$ , inhibition was complete, and a rapid and irreversible sugar leakage resulted (Table 1). PCMB is a more specific inhibitor of  $-\text{SH}$  groups than mercuric ion. At  $10^{-4}\text{M}$ , PCMB caused a 0–10 per cent. reduction in the rate of sugar accumulation, and at  $10^{-3}\text{M}$  (the concentration usually used for inhibition of  $-\text{SH}$  groups) PCMB completely inhibited sugar uptake and caused sugar leakage

(Table 1). Cupric ion (cupric sulphate), which often appears to act in a similar fashion to PCMB, at  $10^{-4}\text{M}$  reduced the rate of sugar accumulation up to 20 per cent., and at  $10^{-3}\text{M}$  caused complete inhibition and induced sugar leakage (Table 1).

(d) *Inhibition by Phloridzin*

Phloridzin, at concentrations completely effective in inhibiting sugar accumulation in animal tissues ( $10^{-6}$ – $10^{-5}\text{M}$ ), had no effect on sugar accumulation in sugar-cane. At  $2 \times 10^{-3}\text{M}$ , phloridzin caused a 10–80 per cent. reduction in the rate of sugar accumulation (Fig. 6).

TABLE 1  
EFFECT OF METABOLIC INHIBITORS ON SUGAR ACCUMULATION IN SUGAR-CANE

Inhibitor	Molarity	Inhibition (%)	Leakage (% uptake)
Azide	$10^{-3}$	100	165
Arsenate	$10^{-3}$	100	128
Iodoacetate	$10^{-3}$	100	173
Mercuric ion	$10^{-6}$	0	—
	$10^{-5}$	5–10	—
	$10^{-4}$	100	37
<i>p</i> -Chloromercuribenzoate	$10^{-5}$	0	—
	$10^{-4}$	0–10	—
	$10^{-3}$	100	39
Cupric ion	$10^{-5}$	0	—
	$10^{-4}$	10–20	—
	$10^{-3}$	85–100	0–20
Phosphate	$2 \times 10^{-3}$	30	—

(e) *Effect of Borate, Phosphate, and Magnesium Ions*

Borate ion (as boric acid,  $5 \times 10^{-4}\text{M}$  and  $1 \times 10^{-3}\text{M}$ ) and phosphate ion (as potassium dihydrogen phosphate,  $2 \times 10^{-3}\text{M}$ ) both failed to stimulate sugar accumulation in sugar-cane tissues, instead causing a reduction (Fig. 7; Table 1). Magnesium ion (as magnesium chloride,  $2 \times 10^{-3}\text{M}$  and  $5 \times 10^{-3}\text{M}$ ) caused a 0–20 per cent. increase in the rate of sugar accumulation (Fig. 7). The concentration of borate used may have been slightly toxic to sugar-cane. Alternatively, there could have been an optimal concentration of boron already present in the control tissue. This was probably the case for magnesium and phosphate.



## IV. DISCUSSION

The effect of the various inhibitors on the sugar-accumulation process in sugar-cane conformed closely to the general pattern of action of inhibitors on many other transport processes in plant and animal tissues. Compared with these, the sugar-accumulation process in sugar-cane was generally sensitive to lower inhibitor concentrations than most plant processes, but less sensitive to inhibitors than the sugar-accumulation process in animal tissues. However, the only major difference found concerned the action of phloridzin. This inhibitor at  $10^{-6}$ – $10^{-5}$ M has been shown by a number of workers to be a specific inhibitor of sugar accumulation in animal tissues: in sugar-cane there was no effect at concentrations as high as  $10^{-4}$ M, and even at  $2 \times 10^{-3}$ M inhibition was not complete. At this concentration phloridzin is believed to act as a general inhibitor of phosphorylation (James 1953*a*). It is

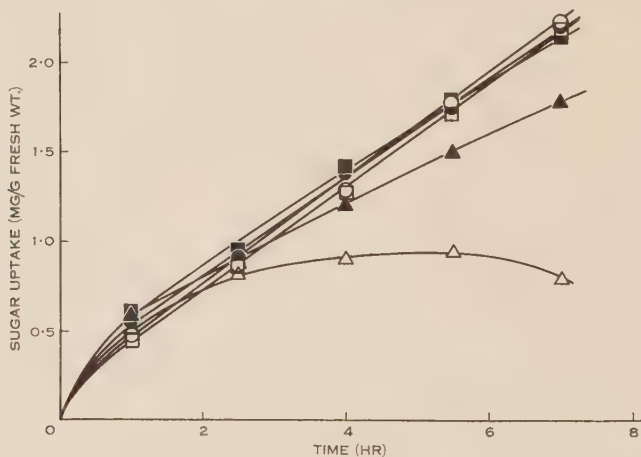


Fig. 6.—Effect of phloridzin on sugar accumulation in immature tissues: ○, ● tissue in 0.25 per cent. glucose (expts. 1 and 2); □, ■ tissue in 0.25 per cent. glucose +  $10^{-4}$ M phloridzin (expts. 1 and 2); △, ▲ tissue in 0.25 per cent. glucose +  $2 \times 10^{-3}$ M phloridzin (expts. 1 and 2). Expt. 1: tissue sample weight 60 g; solution volume 120 ml. Expt. 2: tissue sample weight 50 g; solution volume 115 ml.

possible that no effect was observed at low concentrations because the tissue inactivated the phloridzin. If this were so, however, it would be expected that when dilute solutions of the inhibitor were used, there would be an initial phase when inhibition occurred (as with cyanide, Fig. 5). At higher concentrations the pattern was rather one of increasing inhibition with time. This is more what would be expected if the inhibitor, being of high molecular weight, was hindered in reaching the site of action in the tissue. In view of the rapid action of the other inhibitors, in particular PCMB which also has a high molecular weight, this too seems unlikely. Weatherley (1953) found that phloridzin at a concentration of 0.1 per cent. (*c.*  $2 \times 10^{-3}$ M) did not inhibit sugar uptake in leaf tissues; though there is some doubt as to whether this author was studying a true accumulation process (Pennell and Weatherley 1958). Street and Lowe (1950) found that growth of tomato roots was

inhibited 25 per cent. by  $2 \times 10^{-4}M$  and 67 per cent. by  $2 \times 10^{-3}M$  phloridzin and that the inhibition was partially reversed by increased sucrose concentrations. They attributed the inhibition to an effect of phloridzin on sucrose utilization. Thimann and Marre (1954) found that phloridzin,  $2 \times 10^{-3}M$ , inhibited the elongation of *Avena* coleoptiles, but the inhibition was independent of sugar uptake, and not reversed by the addition of hexose phosphates. In all these cases the effective concentration was high and the degree of inhibition slight as compared with phloridzin inhibition of sugar uptake in animal tissues. It appears that the sugar-accumulation process in plants may differ from that in animals by not being specifically sensitive to phloridzin.

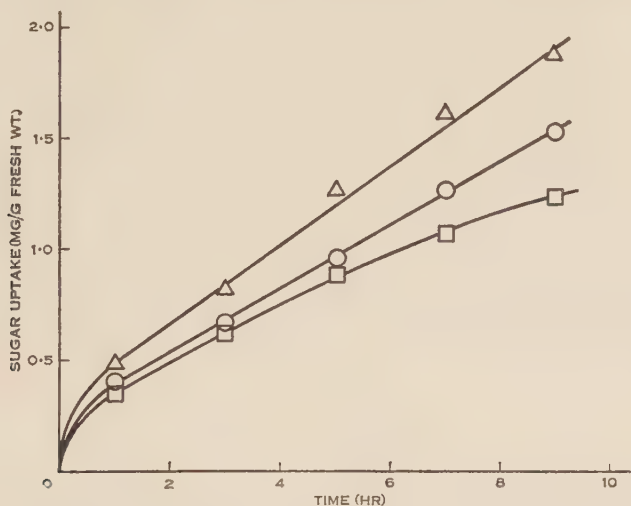


Fig. 7.—Effects of magnesium ion and borate ion on sugar accumulation in immature tissues: ○ 0.275 per cent. glucose; △ 0.275 per cent. glucose +  $2 \times 10^{-3}M$  magnesium chloride. □ 0.275 per cent. glucose +  $5 \times 10^{-4}M$  boric acid. Tissue prewashed for 21 hr in distilled water,  $2 \times 10^{-3}M$  magnesium chloride, and  $1 \times 10^{-3}M$  boric acid respectively. Tissue sample weight 65 g; solution volume 130 ml.

It was found that, in general, when inhibitors were added to the tissue, there was either a very slight inhibition of sugar accumulation, or a complete inhibition and a rapid sugar leakage from the tissue. In some cases the inhibition and leakage could be stopped and accumulation restored by removal of the inhibitor. It is therefore unlikely that the sugar leakage was always, if ever, due to any destruction of cell membranes by the inhibitor. There could have been a sudden increase in the permeability of the tonoplast once a critical inhibitor concentration was reached. However, three distinct types of inhibitors were involved; inhibitors of aerobic respiration (anaerobic conditions (Bialeski 1960), cyanide), inhibitors of phosphate transfer (DNP, arsenate), and inhibitors which act on enzymes containing -SH groups (PCMB, cupric ion, mercuric ion), each of which stopped sugar accumulation and caused sugar leakage when present in a concentration similar to that in which it usually acts on most other processes in plant tissues (James 1953*a*, 1953*b*).

Hence it is unlikely that the sugar leakage was caused by a direct effect of inhibitors on the cell permeability. The evidence suggests that, in the immature storage tissues at least, net leakage of sugar from the vacuole is prevented only by the countering action of an accumulation mechanism which obtains energy from the normal metabolic processes of the cell. Once the inhibitor is present in amounts sufficient to inhibit this accumulation mechanism, sugars pass freely from the cell into the outside solution. The degree of activity of the accumulation system determines whether sugar will be taken into the tissue, held at equilibrium, or lost by leakage. The behaviour of the mature tissue is more uncertain. Anaerobic conditions have been shown to cause sugar leakage in immature tissues, but apparently not in mature tissues, despite the greater concentration of sugars (Bielecki 1960). This suggests that as the cell reaches final maturity it becomes more impermeable and less dependent on the accumulation mechanism for maintaining its internal sugar concentration. Some of the results with DNP inhibition supported this suggestion, since sometimes DNP inhibition resulted in a slower leakage from mature tissue than from immature tissue (e.g. Fig. 2), but occasionally (e.g. Fig. 3) the reverse was found. Variations in time of year, duration of the experiment, and concentration of DNP all affected the relative leakage rates from the two tissues; and the tissue itself apparently modified the action of the inhibitor by slowly inactivating the DNP. Clearly, other methods will have to be sought in order to determine whether or not changes in tonoplast permeability occur in the maturing sugar-cane storage cells.

#### V. ACKNOWLEDGMENTS

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# SOME EFFECTS OF BORON ON ROOT GROWTH

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## Summary

The absence of boron in the root environment reduced the total linear growth of the radicles of the four dicotyledon and one monocotyledon species studied. After growth for 4 days in a boron-free medium, the growth rate of the maize radicle was comparable to that in a plus-boron medium, whilst the growth of the field bean radicle ceased. The minimum boron requirement for the unrestricted growth of the field bean (*Vicia faba* var. *minor*) radicle over 120 hr was 0.005 p.p.m. B. Each microgram of boron in this medium evoked a mean radicle elongation of 51 mm.

Chemical analysis and the growth response of beans upon transfer from plus-boron to boron-free solutions demonstrated that, despite a higher concentration of boron in the radicle tip (as in other young tissues), the reserves of boron in the radicle tip were sufficient to support only about 5 hr of growth at the plus-boron growth rate. Feeding experiments and seed analysis, coupled with growth studies, showed that there was little movement of boron from either the seed to the radicle or from the epicotyl to the radicle tip.

No regrowth occurs from the tips of radicles immersed for 72 hr or more in solutions lacking boron. An analogy is suggested between the effects of boron deficiency and X-irradiation on the bean root tip. The influence of boron deficiency on lignification and differentiation in the bean radicle is discussed.

## I. INTRODUCTION

The growth of roots, either attached to the plant or when excised and grown in sterile culture (Neales 1959b), is severely restricted in the absence of boron in the growth medium. Whittington (1957, 1959) and Scholz (1959) have used the bean radicle for studies of the metabolic effects of boron deficiency. However, they did not investigate the boron requirement for the growth of this root: they used nutrient cultures either without boron or with a boron content (0.5 p.p.m.) in excess of that required for root growth.

This paper reports the results of experiments in which the minimal boron requirement for the growth of the bean and other radicles was examined. The bean radicle was also used in an examination of some physiological aspects of the growth inhibition effected by the absence of borate in the growth medium.

## II. METHODS AND MATERIALS

### (a) Plant Material

The following species were used: field (or tick) bean (*Vicia faba* var. *minor*); broad bean (*Vicia faba* cv. *Leviathan*); garden pea (*Pisum sativum* cv. *Greenfeast*); maize (*Zea mays* cv. *Hickory King*); flax (*Linum usitatissimum* cv. *Ventnor*).

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(b) *Cultural Methods*

The seeds were sterilized in an ethanol-hydrogen peroxide mixture (1 : 1 v/v) for 5 min; they were then washed and soaked for 12 hr in boron-free water. The imbibed seeds were then allowed to germinate for 48 hr on moist vermiculite at 22°C in the dark. The seedlings were then transferred to the culture solutions, when their radicles were between 20 and 30 mm in length. The composition of the culture medium is given in Table 1. The micronutrients and iron were supplied from B.D.H. reagents, A.R. grade. The macronutrients and water were freed from boron contamination by methods described previously (Neales 1959b).

The seedlings were grown in 9-l. polythene containers, on each of which was placed a "Perspex" sheet in which holes 3 mm in diameter were drilled. The radicles passed through these holes into the nutrient solution. Up to 50 plants were grown in each container.

TABLE 1  
COMPOSITION OF THE BASIC CULTURE MEDIUM  
Boron was added, in varying amounts, as  $H_3BO_3$

Macronutrients	Concn. (mg/l)	Microelements	Concn. (p.p.m.)	Microelements	Concn. (p.p.m.)
$Ca(NO_3)_2$	33.6	Fe(as Fe-EDTA*)	0.5	Cu(as $CuSO_4 \cdot 5H_2O$ )	0.0075
$MgSO_4 \cdot 7H_2O$	4.2	Mn(as $MnSO_4 \cdot 4H_2O$ )	0.075	Zn(as $ZnSO_4 \cdot 7H_2O$ )	0.0075
$KH_2PO_4$	6.0	Mo(as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ )	0.0025	I(as KI)	0.0125

\* Ethylenediaminetetra-acetic acid.

Radicle growth took place in a dark room at 22°C. The maximum growth period for any experiment was 170 hr. Radicle measurements were normally made each day by withdrawing the seedling and measuring the length of the radicle against a rectangle of clean, ruled, millimetre graph paper. From these measurements the daily increments of radicle growth were obtained. The radicle growth curves presented in this paper are a plot of the sum of the daily growth increments against time. This method of presentation reduces the variation due to the differences in radicle length at the beginning of each experiment.

(c) *Boron Analysis*

Boron was determined by a modification of the method of MacDougall and Biggs (1952). Root material was washed three times in boron-free water prior to drying at 95°C, weighing, and ashing. The plant ash was acidified and boiled for 2 min with hydrazine sulphate prior to filtration, thus reducing the nitrate in the ash which interferes with this estimation (Hewitt 1952, p. 196). Equivalent quantities of hydrazine sulphate were added to the reagent blanks. The optical density

(O.D.) of the quinalizarin-borate colour was measured at 600 m $\mu$ . A plot of O.D. against added boron was linear between 0.4  $\mu$ g B.

(d) *Translocation Studies using the Field Bean Seedling*

Bean seedlings grown for 48 hr in plus-boron solutions were used, when the epicotyl was approximately 30 mm and the radicle 60 mm long. The epicotyl was then cut off 10 mm above the cotyledons and solutions fed via a glass capillary, which was pushed a distance of 5 mm into the stump of the epicotyl. Decapitation of the epicotyl did not affect radicle growth over 160 hr. Approximately 25  $\mu$ l of

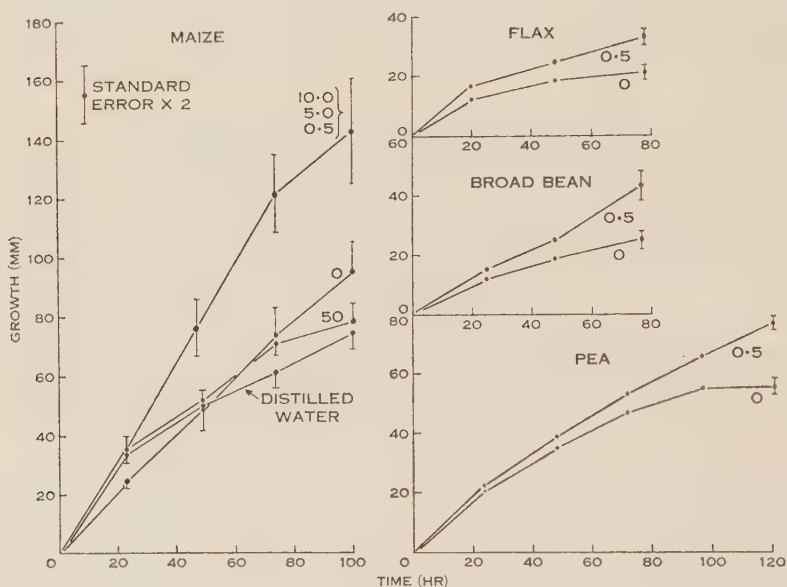


Fig. 1.—Effect of boron on the growth of the radicles of various species. The concentration of boron (in p.p.m.) in the culture medium is indicated near the respective growth curves.

solution was thus fed to each plant, and this was absorbed in 12 hr. Immediately after decapitation and the insertion of the capillary, the plants were transferred to a boron-free solution. The translocation of boron from the epicotyl to the radicle tip, a distance in excess of 70 mm, was assessed by the linear growth of the radicles of fed plants after transfer to the boron-free solution. The transfer of bean plants from a plus- to a minus-boron solution normally inhibits radicle growth after 48 hr (Table 6). After blotting the guttation drop which appeared on decapitation, there was no sign of external leakage of solution from the capillary. The possibility of leakage of boron from the upper part of the bean radicle into the culture solution, and thus supplying the radicle tip with boron, was minimized by renewing the minus-boron culture solution every 12 hr.

## III. RESULTS

Apart from an inhibition or reduction of radicle growth, the absence of boron in the radicle growth medium also induced a curling and swelling of the radicle tip (Plate 1), and differentiation of the stele almost to the tip of the radicle (Plate 1, Fig. 2). These observations accord with those of Warington (1923), Sommer and Sorokin (1928), and Whittington (1957, 1959).

(a) *Effect of Boron on the Growth of the Radicles of Maize, Pea, Broad Bean, and Flax*

The growth of the radicles of all the species studied was significantly reduced by the absence of boron in the growth medium (Figs. 1 and 2). Table 2 indicates the degree to which radicle growth rate was limited, over the last sampling period,

TABLE 2

EFFECT OF THE ABSENCE OF BORON IN THE ROOT ENVIRONMENT ON THE MEAN RADICLE GROWTH RATE OVER THE LAST SAMPLING PERIOD OF EACH EXPERIMENT

Plant	Period of Growth (hr after start of expt.)	Radicle Growth Rate (mm/24 hr)		Growth Rate in Minus-boron Medium as % of Plus-boron Growth Rate	Figure showing Growth Curves
		Plus Boron	Minus Boron		
Maize	74-99	20.5	20.2	98.5	Fig. 1
Field bean	68-95	27.8	0.1	0.4	Fig. 2(a)
Flax	48-78	6.2	3.0	48.4	Fig. 1
Broad bean	48-77	15.1	5.7	37.7	Fig. 1
Pea	97-121	11.1	0.4	3.6	Fig. 1

in boron-deficient media when compared with the growth rate in the presence of boron. It is apparent that the inhibitory effect of a lack of boron in the root environment is greatest in the field bean and least in maize. The lower boron requirement for the growth of the maize radicle, compared to that of the field bean, conforms to the generalization that monocotyledons have a lower boron requirement for growth than the dicotyledons (Bertrand and Silberstein 1941; Marsh 1942; Shkol'nik and Makarova 1949).

There was considerable variation in the radicle growth rate of the species studied. The mean growth over each experiment and the maximum daily growth rate are given in Table 3. The results of experiments with the field bean are included for comparison. Occasionally a growth of up to 70 mm in 24 hr was recorded for the maize radicle.



The growth of the radicle of both the field bean and maize was reduced in distilled water. However, the bean radicle was translucent and flaccid after 24 hr, whilst the maize radicle was slowly growing and of normal appearance after 72 hr in distilled water. It would seem that the maize radicle has a much lower ionic requirement for growth than that of the bean. A similar relationship exists between the boron requirement for radicle growth of the two species. The growth inhibition of the bean radicle by distilled water is identical to that reported by True (1914) for the radicle of *Lupinus albus*.

The radicle of the field bean has a high growth rate (approx. 30 mm per day), is very sensitive to the absence of boron, and grows without the development of lateral roots for 96 hr. This root is thus the most suitable of those examined for

TABLE 3  
RADICLE GROWTH RATES IN PLUS-BORON SOLUTIONS

Plant	Duration of Experiment (hr)	Total Growth (mm)	Mean Growth Rate (mm/24 hr)	Period (hr)	Duration (hr)	Total Growth (mm)	Maximum Growth Rate (mm/24 hr)
Maize	72	129.6	43.2	24-48	24	50.1	50.1
Field bean	95	109.5	27.7	44-68	24	29.8	29.8
Broad bean	77	43.3	13.5	48-77	29	18.3	15.1
Flax	78	32.5	10.0	0-24	24	16.9	16.9
Pea	121	77.2	15.2	0-24	24	22.2	22.2

the study of boron requirement of roots. The remainder of this paper describes experiments in which the growth of the field bean radicle was studied in relation to the boron concentration in the external medium.

(b) *Boron Requirement for the Growth of the Field Bean Radicle*

The effect of the following boron concentrations on the growth of the field bean radicle was studied: 0, 0.5, 5.0, 10.0, and 50 p.p.m.; in addition, the growth of radicles in distilled water was investigated. The results of this experiment are given in Figure 2(a). The growth data for the 0.5, 5.0, and 10.0 p.p.m. boron treatments were statistically indistinguishable and were therefore pooled.

It is apparent from these data that a wide range of boron concentrations (0.5-10.0 p.p.m. B) supports a similar and high radicle growth rate. 50 p.p.m. B restricts, but does not inhibit, the growth of the bean and maize radicle. The growth of the bean radicle is inhibited after 48 hr in a growth medium lacking boron; this is similar to Whittington's (1957, 1959) results.

In a second experiment the growth of the bean radicle in media containing 0, 0.0005, 0.0025, 0.005, 0.05, and 0.5 p.p.m. B was studied. The results are given in Figure 2(b).

It is evident that the growth of the radicle over 100 hr is not restricted by lack of boron in concentrations above 0.005 p.p.m. At the end of the experiment all radicles growing in solutions of boron concentration of 0.0025 p.p.m. and below had the characteristic visual symptoms of boron deficiency (Plate 1). This was also true of a proportion of those radicles in 0.005 p.p.m. B. The growth of the radicles in 0.005 p.p.m. B from 120–168 hr was significantly less ( $P < 0.05$ ) than those in 0.5 p.p.m. B. It appeared, therefore, that the total boron supplied to

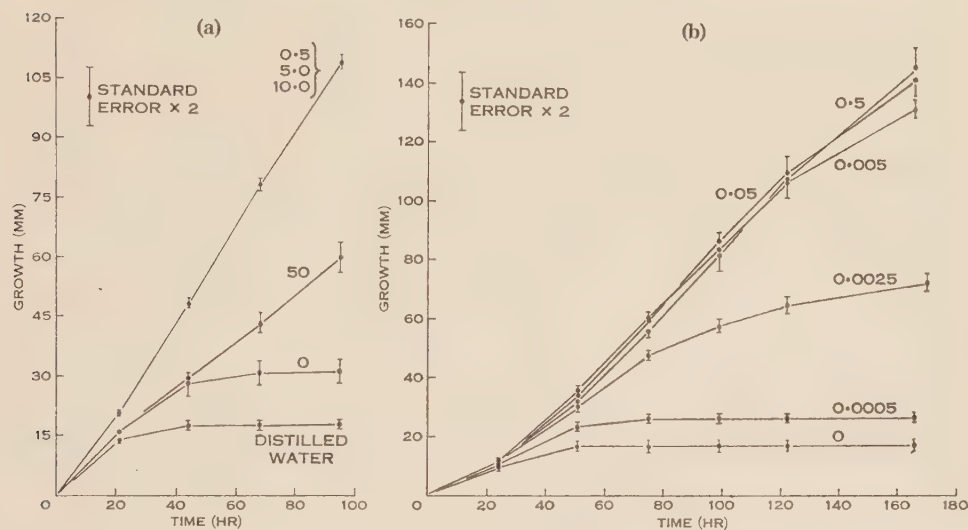


Fig. 2.—(a) Effect of 0–50 p.p.m. boron, and (b) of 0–0.5 p.p.m. boron on the growth of the field bean radicle.

the plants in the 0.005 p.p.m. boron treatment (45  $\mu\text{g}$ ) was just sufficient to support the growth of 20 plants for 168 hr. From these data it is possible to calculate the root growth response to the amount of boron supplied to the culture medium (Table 4).

From this table it is apparent that the maximum root growth response per unit amount of boron in the culture medium (51.0 mm growth per  $\mu\text{g}$  B supplied) occurs in the 0.005 p.p.m. B treatment. Similar root growth increments per  $\mu\text{g}$  B supplied are obtained in nutrient cultures of boron concentration lower than 0.005 p.p.m.; although, due to a lower boron content, root growth ceased before 168 hr (Fig. 2(b)). In the solutions of 0.05 and 0.5 p.p.m. B the root growth response (5.3 and 0.6 mm per  $\mu\text{g}$  B supplied) is smaller, indicating an incomplete utilization of the excess boron present in the medium.

Assuming a root growth extension response of 51.0 mm per  $\mu\text{g}$  B supplied, the mean extension growth of 17.6 mm in 0 p.p.m. B solution (Table 4) represents

a boron requirement of  $0.35 \mu\text{g B}$  per plant. If there was no boron contamination in this medium, this  $0.35 \mu\text{g B}$  is presumably derived from the bean cotyledons.

TABLE 4  
BORON REQUIREMENT FOR THE GROWTH OF THE FIELD BEAN RADICLE

Concentrations of Boron in Nutrient Medium (p.p.m.)	Boron Added to 9 Litres of Solution ( $\mu\text{g}$ )	No. of Plants per Treatment	Mean Growth per Plant (mm)	Mean Increase in Root Length minus Increase in 0 p.p.m. Boron (mm)	Increase in Root Length per $\mu\text{g}$ Boron Supplied (mm)
0	0	20	17.6	—	—
0.0005	4.5	20	26.6	9.0	39.1
0.0025	22.5	20	72.5	54.9	48.6
0.005	45	20	132.3	114.7	51.0
0.05	450	19	142.5	124.9	5.3
0.5	4500	20	146.6	129.0	0.6

Chemical analysis of a sample of imbibed beans and also of the testas of 5-day-old bean seedlings (Table 5) indicated that the boron content of the testa ( $0.9$

TABLE 5  
BORON CONTENT OF THE FIELD BEAN SEED

Stage of Development	Portion of Seed Analysed	Number	Dry Wt. (g)	Total Boron Content ( $\mu\text{g}$ )	Boron Content per Seed ( $\mu\text{g}$ )	Boron Concn. (p.p.m.)
Ungerminated but imbibed seed	Cotyledons, plus plumule and radicle	50	18.278	65.00	1.30	3.56
	Testa	50	2.900	43.75	0.88	15.09
	Complete seed	50	21.178	108.75	2.18	5.14
5-day-old seedlings	Testa	25	1.520	23.52	0.94	15.47

$\mu\text{g B}$  per seed) is not available to the seedling during 5 days of growth. Thus the maximum amount of boron from the seed available for seedling growth is repre-

sented by that present in the cotyledons and embryo ( $1.3 \mu\text{g B}$  per seed). These values for the boron content of beans are similar to those reported by Owen, Snow, and Thom (1945), but differ from those of McLean and Hughes (1936) who used a much older analytical technique.

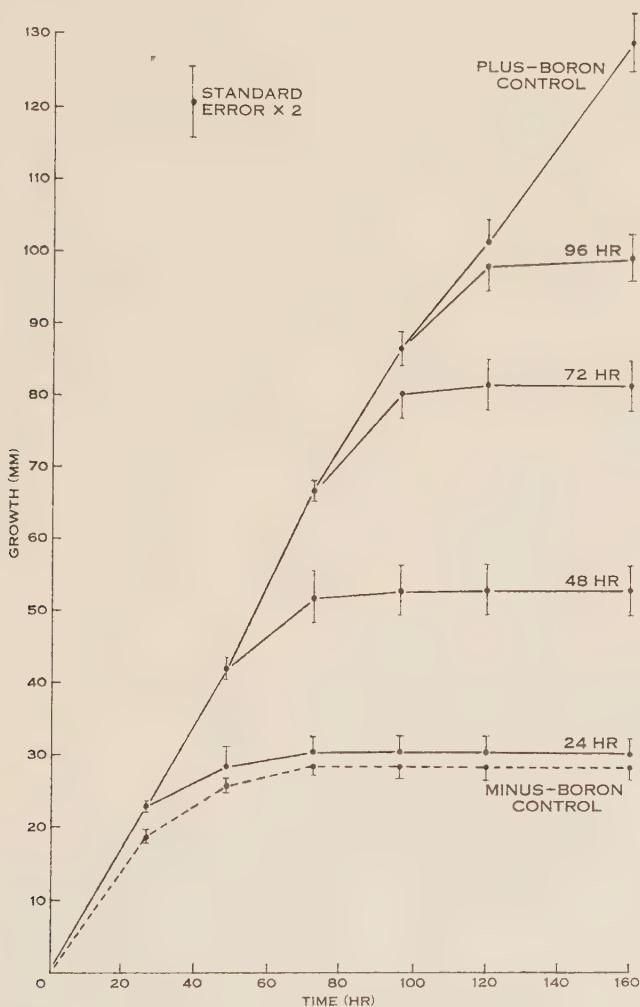


Fig. 3.—Effect of transfer from plus-boron ( $0.5 \text{ p.p.m.}$ ) to minus-boron solutions on the growth of the field bean radicle. Times at which plants were transferred are indicated.

(c) *Effect on Radicle Growth of Transferring Beans from Plus-boron to Minus-boron Solutions and vice versa*

A large sample of beans was grown in either  $0.5 \text{ p.p.m.}$  or  $0 \text{ p.p.m. B}$  solutions. At varying times from the beginning of the experiments beans supplied with boron in the culture medium were transferred, after washing the radicles in water,



to minus-boron cultures. The converse of this experiment was also done. Radicle lengths were measured periodically before and after the transfer.

(i) *Effect of Transferring Beans from 0.5 to 0 p.p.m. B Solutions.*—The results of this experiment are given in Figure 3. Despite the fact that the boron content of the medium was approximately 100 times that necessary for growth, it is apparent that the removal of boron from the root environment causes a very rapid inhibition of growth, and this is independent of the time at which the transfer is made (Table 6). It is apparent therefore that, under these conditions, the radicle is unable to maintain a physiologically active internal supply of boron. The increments of radicle growth in the 48 hr after transfer to minus-boron solutions are given in Table 6.

TABLE 6  
RADICLE GROWTH IN THE FIRST AND SECOND 24-HR PERIOD AFTER TRANSFERRING BEAN SEEDLINGS FROM PLUS- TO MINUS-BORON SOLUTIONS

Time of Transfer	No. of Beans	Mean Growth (mm) after Transfer to Minus-boron Solutions		No. of Beans	Mean Growth (mm) of Beans not Transferred	
		First 24 Hours	Second 24 Hours		First 24 Hours	Second 24 Hours
At 24 hr	10	6.4	1.7	48	19.1	24.9
At 48 hr	10	8.8	0.5	38	24.9	19.9
At 72 hr	9	6.6	1.4	29	19.9	15.0
At 96 hr	10	4.7	1.3	19	15.0	14.5

Radicles transferred after growth for 72 hr in a 0.5 p.p.m. B solution grew a further 8.0 mm in the minus-boron solution. This growth corresponds to a "carry over" of 0.16  $\mu\text{g}$  of physiologically active boron, assuming that 1  $\mu\text{g}$  B evokes a radicle elongation response of 51.0 mm (Table 4). This estimate of the amount of boron in the radicle tip was compared with that found by chemical analysis of the radicles of beans grown for 72 hr in nutrient solutions containing 0.5 p.p.m. B (Table 7). It was found that the boron present in each radicle tip was approximately 0.04  $\mu\text{g}$ , and that each whole radicle contained approximately 0.1  $\mu\text{g}$  B. These results are further considered in Section IV.

(ii) *Effect of Transferring Beans from a Minus- to a Plus-boron (0.5 p.p.m.) Solution.*—The results of this experiment are given in Figure 4. These data indicate that the radicle apical meristem is irreversibly damaged if immersed for 72 hr or more in a minus-boron medium. Radicles grown for 24 hr in a minus-boron medium and then transferred to a plus-boron medium have a subsequent growth indistinguishable from the plus-boron control treatment. The effect of transfer from

a minus- to a plus-boron medium at 48 hr has an effect intermediate between the results of transfer at 24 and 72 hr.

The large variability in the mean length of the radicles transferred at 48 hr may be attributed to the fact that one radicle failed to grow after transfer to the

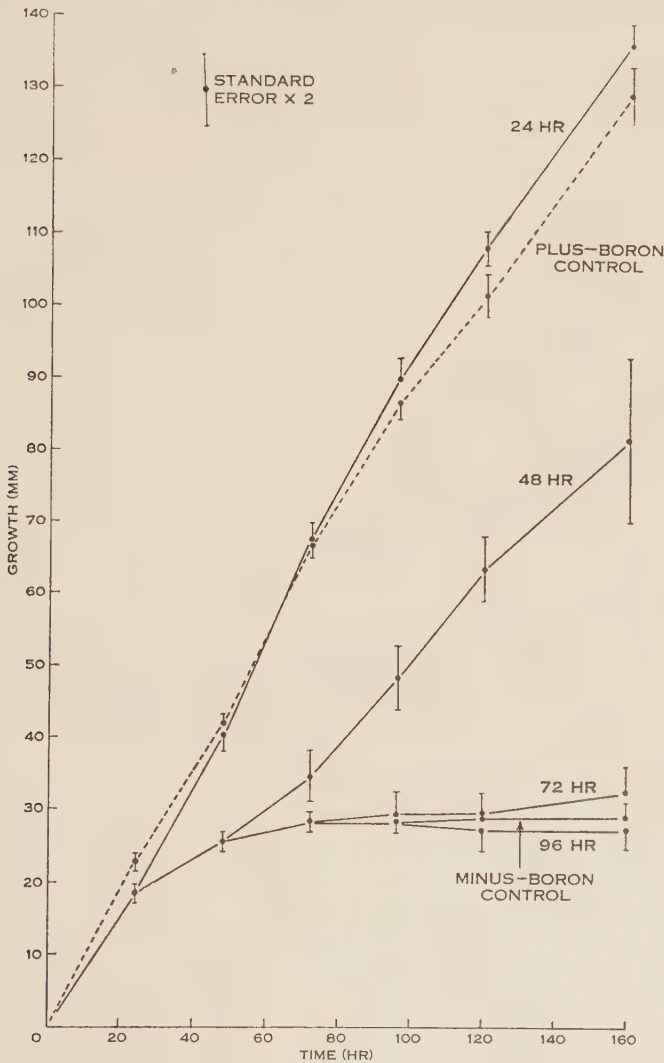


Fig. 4.—Effect of transfer from minus-boron to plus-boron (0.5 p.p.m.) solutions on the growth of the field bean radicle. Times at which plants were transferred are indicated.

plus-boron solution; the remainder recovered and from day 5 to day 7 grew as rapidly as the plus-boron controls. The regrowth of radicles after 72 hr in a solution lacking boron was apparent only in four radicles, and was evident only in the last day of the experiment. Examination showed that this regrowth proceeded from a

lateral root initial, which can be seen to be present almost to the tip of an old boron-deficient root (Plate 1, Fig. 4). After 48 hr in a minus-boron solution, the regrowth of roots was apparently terminal, but a constriction behind the meristem

TABLE 7

BORON CONTENT OF THE RADICLE TIPS OF BEANS GROWN FOR 72 HR IN A NUTRIENT SOLUTION CONTAINING 0.5 p.p.m. BORON

Portion of Radicle	No. in Sample	Fresh Weight (mg)	Dry Weight (mg)	Boron Content per Radicle ( $\mu$ g)	Boron Concentration (p.p.m.)		
					Fresh Weight Basis	Dry Weight Basis	Root Volume Basis
Experiment 1							
20-mm tip	47	709.4	43.9	0.035	2.29	37.1	2.75
Remainder of radicle	57	8330.4	498.3	0.056	0.38	6.4	—
Total				0.091			
Experiment 2							
15-mm tip	70	836.1	54.9	0.044	3.71	56.5	4.61
Remainder of radicle	60	9582.7	552.7	0.056	0.35	6.1	—
Total				0.100			

was initially apparent (Plate 1, Fig. 4). A similar feature has been illustrated in the boron-deficient pea root tip (Sommer and Sorokin 1928, plate VII, fig. 2),

TABLE 8

EFFECT OF FEEDING SUCROSE AND BORIC ACID THROUGH THE EPICOTYL ON THE GROWTH OF BEAN ROOTS

Boron Content of Growth Medium (p.p.m.)	Solution (vol. 25 $\mu$ l) Fed via Epicotyl	Root Growth after 74 Hr (mm)
0.5	—	$75.5 \pm 3.7$
0	Water	$13.0 \pm 2.6$
0	50 $\mu$ g boron	$37.8 \pm 3.5$
0	3000 $\mu$ g sucrose	$13.8 \pm 1.3$
0	{ 50 $\mu$ g boron 3000 $\mu$ g sucrose	$32.7 \pm 4.1$

and also in the tip of the bean root after irradiation with X-rays (Gray and Scholes 1951). Thus, it is most probable that the bean root terminal meristem is damaged if boron is absent from the root environment for periods above 24 hr.

*(d) Translocation of Boron from the Epicotyl to the Radicles of the Bean*

Since the lack of boron has such a marked inhibition on the growth of the bean radicle, and also because the site of this inhibition resides in the radicle meristem, the bean seedling is a most suitable object for the study of boron translocation. The methods used in this experiment have been described earlier.

Eight plants were allocated to each of five treatments (Table 8). At zero time the plants were fed with various solutions through their epicotyls, the radicles were washed in boron-free water, and they were then transferred to nutrient media

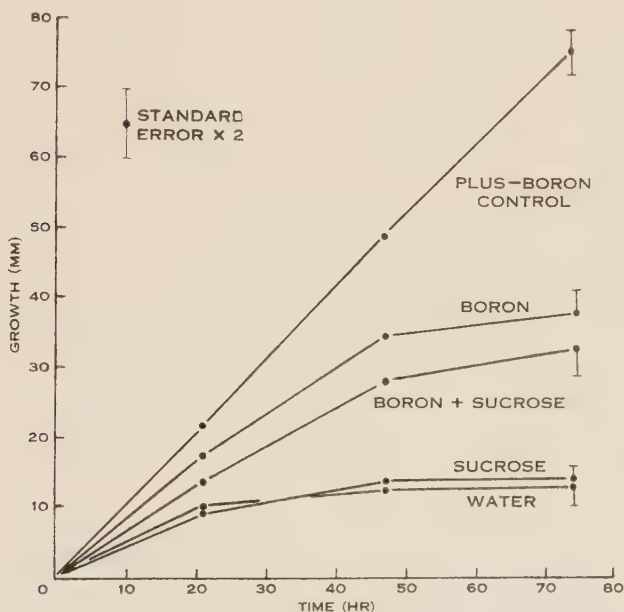


Fig. 5.—Effect of feeding sucrose and boron (as  $H_3BO_3$ ) via the epicotyl on the growth of the field bean radicle in a boron-deficient medium. The plus-boron control plants were growing in a medium containing 0.5 p.p.m. boron (see Table 8).

lacking boron. The radicle growth of each plant was then individually followed for 74 hr. The radicle growth curves are given in Figure 5 and accumulated radicle growth of each treatment in Table 8.

These data demonstrate that the inhibition of radicle growth in minus-boron solutions is not alleviated by feeding sucrose through the epicotyl. The radicle of beans in the 0.5 p.p.m. B solutions (in which  $45 \mu g$  B was available to each plant) had a uniform and normal growth rate for 74 hr (Fig. 5), whereas  $50 \mu g$  boron fed through the epicotyl of each plant growing in minus-boron solutions did not prevent either a large decrease in radicle growth rate or the eventual appearance of boron deficiency symptoms in the radicle tips. It is apparent that there is a very limited movement of boron from the epicotyl to the radicles.



## IV. DISCUSSION AND CONCLUSIONS

(a) *Boron Concentration Necessary for Root Growth*

The results of the first experiments reported in this paper consist of a quantitative description of the degree to which the radicle growth of several species is dependent upon an external supply of boron. In the field this potential restriction of root growth is rarely manifest because most soils contain adequate available boron for plant growth (Swaine 1955). Haynes and Robbins (1948) have reported that both calcium and boron in the root environment are indispensable for root growth.

The smallest concentration of boron in the root environment that supported the normal growth of the bean radicle over 168 hr was 0.005 p.p.m. boron (Fig. 2). This is 200 times less than the boron concentration used by Whittington (1957, 1959) in his "plus boron" series, and is similar in magnitude to the minimal boron requirement for the growth of broad beans (Brenchley and Warington 1927) and flax (Neales 1959a).

(b) *Effects of Boron Deficiency on Root Differentiation*

The significance of the advance of lignification and stelar differentiation almost to the tip of the boron-deficient radicle tip (Plate 1, Fig. 2) may be interpreted in at least two ways: (1) Whittington (1957) has demonstrated the inhibition of cell division in the bean radicle tip by lack of boron in the growth medium. If it is assumed that the normal differentiation of the primary stelar structure is not altered by boron-deficiency, then it is possible to envisage the formation of a lignified stele almost to the tip of the radicle, as indeed is the case in boron-deficient radicles. (2) On the other hand, it has been established that boron-deficient plants contain a higher peroxidase (Nason, Oldewurtle, and Propst 1952; Odhnoff 1957) and polyphenol oxidase (Reed 1947; MacVicar and Burris 1948; Klein 1951) activity than normal plants. Furthermore, boron-deficient plants have been shown to accumulate caffeic and chlorogenic acids (Perkins and Aronoff 1956), the former of which has been shown (McCalla and Neish 1959) to be a precursor of lignin. Thus it is possible that lignin formation is actually *enhanced* in boron-deficient bean roots. This possibility also reopens the question of the effect of boron deficiency on the auxin status of plants (Eaton 1940). Jensen (1955) has demonstrated that lignification and peroxidase activity in the bean root could be increased by treatment with indolylacetic acid (IAA), and Torrey (1953) recorded similar effects of IAA on lignification in isolated pea root tips. Thus one possible explanation of this effect of boron deficiency on lignification and peroxidase activity is that boron-deficient roots contain high concentration of an auxin identical, or similar in action, to IAA. This hypothesis is the opposite of that of Eaton (*loc. cit.*) who attempted to reverse the effects of boron deficiency by supplying plants with exogenous IAA.

If one of the functions of boron in plants concerns the orderly regulation of differentiation and lignification (as discussed above), then the observation of Brown, Wright, and Neish (1959), that monocotyledons possess a lignin biosynthesis pathway not possessed by dicotyledons, may afford a possible reason for the much lower boron requirement for the growth of the maize radicle compared to that of

the dicotyledonous species (Table 2). It is apparent that a study of the effects of boron deficiency on the phenolic acid pool in plants (McCalla and Neish 1959) is most desirable.

(c) *Mobility of Boron in the Bean Seedling*

Vascular plants can grow normally in media of very low boron content. It is apparent therefore that the initial distribution of boron, from the external medium to all parts of the plant, must be accomplished without restriction. However, under boron-deficient conditions there is good evidence (Skok 1957a) that redistribution of boron after absorption into sunflower seedlings is either lacking or of small extent. The data given in this paper for the bean seedling support this conclusion. The linear radicle growth of bean seedlings, planted into a medium deficient in boron when their radicles were 20 mm long, was 17.6 mm after which growth ceased (Fig. 2). This is equivalent (Table 4) to the radicle growth-promoting effect of  $0.35 \mu\text{g B}$ , whereas the cotyledons of each bean contained  $1.30 \mu\text{g B}$  (Table 5). Thus only 27 per cent. of the cotyledonary boron is available for radicle growth. The limited movement of the boron from the cotyledons to the roots of bean plants has also been reported by McLean and Hughes (1936). The experiments reported above on the degree to which excess boron fed to the hypocotyls moves to the radicles of bean seedlings also demonstrated that boron did not move readily from epicotyl to radicle tip (Table 8; Fig. 5). In fact, the stimulation caused by feeding  $50 \mu\text{g B}$  (with or without sucrose) was equivalent to the movement to the radicle tip of a "growth activity" equivalent to only  $0.4 \mu\text{g B}$ . A similar restricted growth response to boron solutions injected into plants is recorded by Maier (1941).

(d) *Boron Utilization and Storage by the Radicle Tip*

There was a rapid inhibition of radicle growth upon the transfer of bean seedlings from a plus- to a minus-boron culture medium (Fig. 3). This inhibition indicated that there was a rapid conversion of the physiologically active boron (Skok and McIlrath (1958) demonstrated this to be the dialysable fraction of the boron in plants) to the inactive form, and hence the need for a continuous supply of this element for unrestricted root growth. This indicated that either boron could not be accumulated in any quantity by the radicle tip (where it exerts its growth-promoting effects (Whittington 1957)) or the rate of utilization is so high that the tip becomes rapidly deficient in "active" boron. It was found (Table 7) that the mean boron content of 15 mm of radicle tips of plants growing for 72 hr in  $0.5 \text{ p.p.m. B}$  was  $0.044 \mu\text{g B}$ . This quantity of boron has a growth potential (Table 4) of approximately 2.5 mm. However, the growth over 24 hr of these radicles after washing and transfer to minus-boron solutions was 8.0 mm (Table 6). It would appear, therefore, that the limited radicle growth after transfer from the  $0.5 \text{ p.p.m. B}$  solution is in excess of that to be expected from the boron content of the radicle tip. This "excess" growth, which equals  $8.0 - 2.5 = 5.5 \text{ mm}$ , is equivalent to approximately  $0.1 \mu\text{g B}$  (Table 4), and may be attributed either to a slight carry-over of boron from the plus-boron solution or to the limited translocation of boron from the upper portion of the radicle to the radicle tip. Thus

despite the fact the roots were growing in a solution whose boron content (0.5 p.p.m. B) was 100 times in excess of the minimal requirement, the radicle *tips* did not contain enough boron (assuming it to be all available for growth) to support normal growth for more than a few hours. In terms of root volume the radicle tip concentration of boron is only 5.5 times that of the external medium. These results point to the fact that in the growing radicle tip there is an extremely rapid absorption and utilization of boron, with a very low internal reserve indeed. It is of note that the boron concentration in 15 mm of radicle tips (Table 7) is approximately 10 times that in the remainder of the radicle. This is consistent with the observations of Bertrand and Silberstein (1944, and previous publications listed therein) and McLean and Hughes (1936), who reported higher concentrations of boron in meristematic and embryonic, compared to older, differentiated plant tissues.

#### (e) *Boron and Cell Division*

Whittington (1957) has recorded that in the absence of boron mitotic division in the bean radicle meristem ceases. Many authors (see, for instance, Warrington 1926; Sommer and Sorokin 1928; Jolivet and Walker 1943; and Vial, Carlton, and Strang 1957) have suggested that boron is necessary for cell division in both primary and secondary meristems in many different plants. Experiments reported in this paper (Fig. 4) indicate that in radicles grown for 72 hr in a minus-boron medium, no revival of growth and cell division takes place upon transfer to plus-boron solutions. The regrowth of the root after 48 hours in a minus-boron medium (Plate 1, Fig. 4) is similar in appearance to the type of regrowth obtained after X-irradiation of bean meristems (Gray and Scholes 1951), which Clowes (1959) has suggested are chimerical in nature. This chimera, Clowes suggests, is due to the commencement of division by cells of the pro-meristem that were previously quiescent. This indication that it is the dividing cells that are most damaged by a lack of boron is consistent with the conclusions of Skok (1957*b*) who demonstrated that X-irradiation damage to sunflower seedlings is reduced if boron is removed from the root environment of these plants for a period prior to irradiation.

Although it is improbable that the absorption of X-irradiation by plant tissues will be related to their boron content, it is interesting to note (Conger and Giles 1950; Locksley and Sweet 1954) the direct relationship between radiation damage to living tissues by thermal neutrons and the boron content of these tissues. This is due to the fact that slow neutrons cause the naturally occurring isotopes  $^{10}\text{B}$  and  $^{11}\text{B}$  to emit ionizing radiation. A high proportion of the damage done to *Tradescantia* anthers can be attributed to the neutrons absorbed by the boron in this tissue, despite the fact that the boron content of this tissue was 2.9 p.p.m. (fresh weight basis) (Conger and Giles 1950). Thus since bean radicle tips contain more boron than the rest of the radicle (Table 7), irradiation by slow neutrons will engender damaging radiation especially in the vicinity of those cells (the region of cell division and early differentiation) which are most sensitive to such irradiation (Gray and Scholes 1951; Clowes 1959). These considerations give some radiobiological importance to the distribution of boron in plants.



## V. ACKNOWLEDGMENTS

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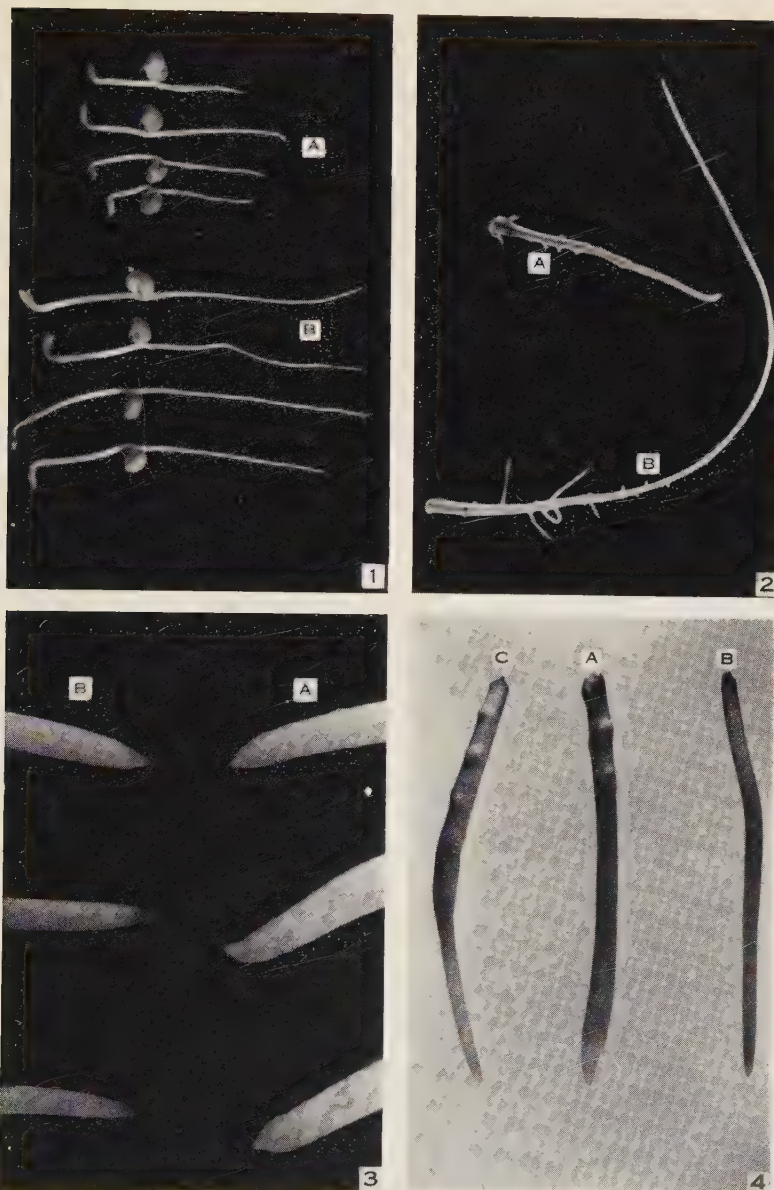


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#### EXPLANATION OF PLATE I

- Fig. 1.—Bean seedlings grown for 76 hr in the presence (*B*) or absence (*A*) of boron.  $\times 0.25$ .
- Fig. 2.—Bean radicles grown for 120 hr in the presence (*B*) or absence (*A*) of boron, and subsequently cleared and stained with acid phloroglucinol. Note the extent of lignification in *A* compared with *B*.  $\times 0.4$ .
- Fig. 3.—Pea root tips after 72 hours' growth in the presence (*B*) or absence (*A*) of boron. Note the swollen root and enlarged root cap of *A*.  $\times 6$ .
- Fig. 4.—The tips of bean roots grown for 96 hr in the presence (*B*) or absence (*A*) of boron. *C* is a bean root tip grown for 48 hr in a minus-boron solution, followed by 48 hr in a solution containing 0.5 p.p.m. boron.  $\times 1.75$ .

## EFFECTS OF BORON ON ROOT GROWTH





# EFFECTS OF NaCl ON THE ION UPTAKE AND GROWTH OF *ATRIPLEX* *VESICARIA* HEWARD

By R. F. BLACK\*

[Manuscript received March 16, 1960]

## Summary

*Atriplex vesicaria*, a xerophytic perennial pasture species of inland Australia, was found to have an extraordinary high tolerance to saline water cultures; seedlings were successfully established in solutions up to 1M NaCl.

The internal salt levels of the mature leaves, when calculated on a leaf water basis, maintained a positive gradient to the culture solution concentrations of about 12 atm over the whole NaCl range. It is shown that the main centres of NaCl accumulation were in the rapidly developing young leaves, but that sufficient NaCl absorption occurred in the mature leaves to maintain comparable levels of chlorine concentration. Only sodium levels increased with leaf maturation, possibly as a response to the Donnan effects of organic anions.

Relatively low levels of NaCl were found in the roots and it was apparent for this species that the NaCl absorption mechanism of the root system serves mainly in uptake and transport of ions to the leaves.

To explain K<sup>+</sup> and Na<sup>+</sup> uptakes in *Atriplex* species, two different mechanisms are suggested for the absorption of alkali cations; firstly, a sodium mechanism where K<sup>+</sup> ions can compete when Na<sup>+</sup> concentrations are low, resulting in "luxury" uptake levels of potassium; and secondly, a potassium mechanism which is completely independent of competition from Na<sup>+</sup> ions. From K/Na ratios of less than unity for uptake from equimolar solutions, it is concluded for the leaves of *A. vesicaria* that a majority of alkali cation carriers must be eminently suitable for sodium absorption, even though a smaller proportion may be completely specific for potassium.

Functionally, luxury potassium levels are considered to be substituting for sodium and not the reverse. Apart from trace-element effects, the specialized sodium uptake of the Chenopodiaceae is looked upon as primarily osmoregulatory in function and not nutritional.

## I. INTRODUCTION

This work continues a study on the comparative physiology, within the one genus, of a typical halophyte, *Atriplex hastata* L., found in coastal salt-marsh and strand communities, and a typical xerophyte, *A. vesicaria* Heward, which is not normally found on saline soils (Osborn and Wood 1923). The work on *A. hastata* has already been presented (Black 1956a) and the autecologies of the two species have been fully discussed (Black 1956b).

Hitherto high internal salt levels have been chiefly associated with halophytes, and species required for high sodium or chlorine studies have usually been drawn from this ecological group. However, many halophytes have morphological or systematic connections with xerophytes; the genus *Atriplex* is such a wide-spanning group.

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Physiological similarities between halophytes and xerophytes were postulated early by Schimper (1903) but these claims fell into disfavour as useful generalities when they failed to explain later experimental facts obtained from studies of divergent ecological mechanisms (Braun-Blanquet 1932, p.192). More recently Magistad (1945) noted the apparent close relationships between many salt-tolerant and drought-resistant plants.

It is probable that among the varied forms which xerophytes are known to take, there are many where a degree of salt tolerance is either absent or plays no important part in their ability to resist, endure, or evade drought periods. However, for the xerophytic *Atriplex* species, where (1) it is known that many have a high salt content in the leaves (Wood 1925; Beadle, Whalley, and Gibson 1957); (2) the genus includes many known halophytes, and (3) their soils often have only a small margin below the borderline of salinity, it would appear that their salt-uptake properties play an essential part in their drought resistance.

## II. METHODS

### (a) *Growth Media*

The basic culture solution, solution 1 of Hoagland and Arnon (1938), was prepared from A. R. reagents and distilled water; NaCl was added to give the series 0, 0.006, 0.02, 0.05, 0.1, 0.2, and by 0.1 increments to 1.0M.  $\text{Na}^+$  and  $\text{K}^+$  were equimolecular in the 0.006M NaCl solutions. The solutions were aerated with a hand bubbler at weekly intervals when water losses were replaced. The 4-l. enamelled cans were covered with drilled sheets of glass which had white paste-board covers on their upper surfaces. In the equilibrium experiment the NaCl concentrations were in two series, A and B.

### (b) *Plant Culture*

The seeds were collected at Fowler's Gap (approx. 70 miles north of Broken Hill, N.S.W.) from bushes with the typical bladder type of fruit. Young seedlings at the cotyledon stage were taken from sand cultures and set out in NaCl treatments up to 0.2M. The plants required for the higher NaCl concentrations were moved up the series at 2-4-day intervals.

The tolerance of these seedlings to NaCl was greater than those of a different seed batch used for preliminary trials (Black 1956a) as seedlings were progressively established in solutions up to 1M NaCl. Even at this stage it appeared that it might be possible to use higher concentrations, but as many plants had already died in the higher concentrations and there was a limited number of plants available, no concentrations higher than 1M were used. The plants were thinned out to one per can, except the duplicate plants of the three highest NaCl treatments.

### (c) *Sampling*

The sampling and harvesting of the equilibrium experiment was carried out in November after 20 weeks of growth in the water cultures. The plants were brought into the laboratory early in the morning, and healthy full-sized leaves

without petioles were removed from about the fourth or fifth node pair from the terminal buds. Young leaves, with the petioles removed as much as possible, were cut from the first pair of nodes from the terminal buds. These ranged from one-quarter to two-thirds of the length of the mature leaves. These samples were weighed fresh, and then washed for  $1\frac{1}{2}$  min in distilled water to remove surface chlorides. The stems were cut at the cotyledon scars, and the roots rinsed three times with distilled water. It was not possible to obtain a sufficiently accurate fresh weight for the root systems.

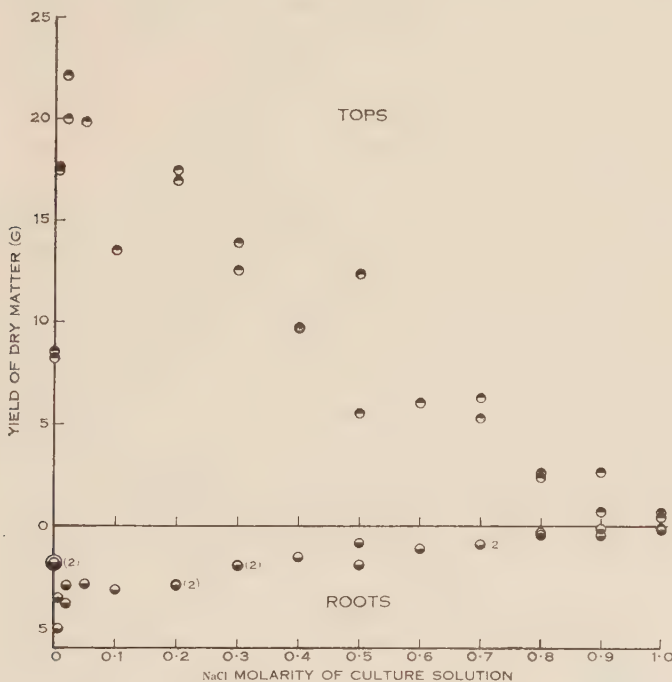


Fig. 1.—Dry matter yields of the roots and tops of *A. vesicaria* plants from the NaCl series, plotted against external NaCl concentrations.

All plant parts were dried overnight in a forced draught oven at  $80^{\circ}\text{C}$  and then weighed oven dry. The water content/dry matter ( $W/D$ ) ratios were calculated for the leaf samples. Samples of all nutrient solutions were analysed for chlorides.

In the dynamic accumulation experiment single plants were grown in  $0.006\text{M}$  NaCl culture solutions for 16 weeks. Immediately after the initial sampling in November, all plants were removed from their cans, their root systems thoroughly rinsed with distilled water, and were then placed in  $0.1\text{M}$  NaCl culture solutions. Plants 1 and 2 were sampled for young and mature leaves while plants 3 and 4 had portions of their root systems sampled. Sampling was carried out in the glass-house early each morning and each leaf sample was collected randomly from the plant, although no stem was sampled of young leaves more than once. Sampling was continued over a period of 10 days from the culture solution transfer.

## (d) Analysis

The samples of young and mature leaves, and the younger portions of the root systems, were all ground in a pestle and mortar. Portions of these samples were dried in an oven at 100°C for 2 hr. The oven dry weight was then determined,

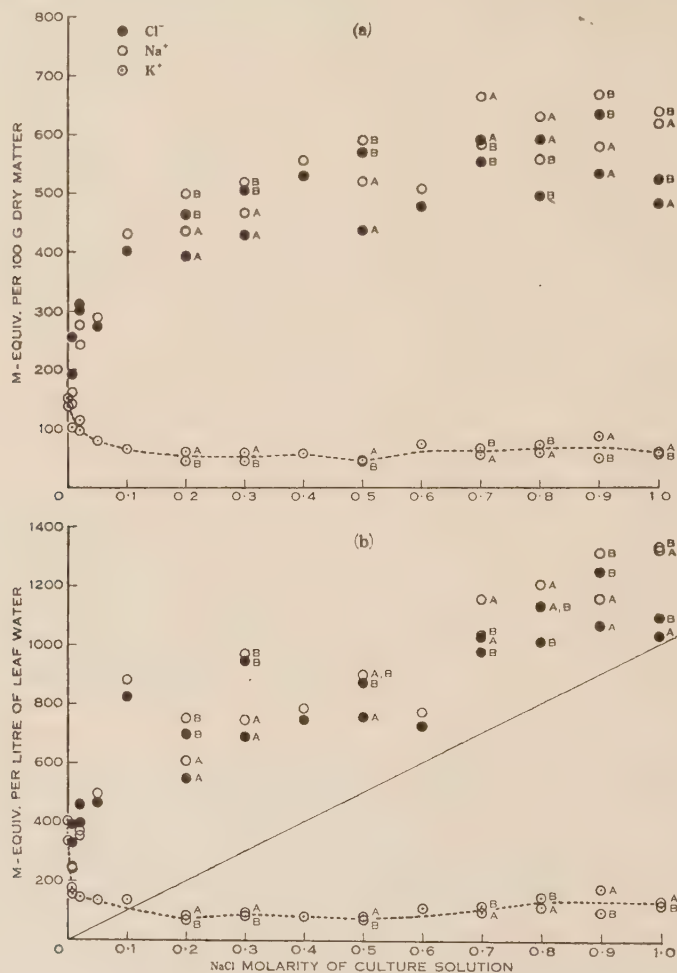


Fig. 2.— $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$  contents of young leaves of *A. vesicaria* plotted against external NaCl concentration.  $\text{Na}^+$  and  $\text{Cl}^-$  contents for 0 NaCl treatments for the two series of plants respectively are (a)  $\text{Na}^+ = 2.6, 3.4$ ;  $\text{Cl}^- = 9.1, 8.5$ ; (b)  $\text{Na}^+ = 7.6, 7.5$ ;  $\text{Cl}^- = 26.7, 18.9$ .

and the material boiled for 1 hr in distilled water in a ratio of approximately 1 : 500. After cooling and filtering, the extract was made up to a known volume at about 1 : 1000.

Sodium and potassium concentrations were determined using a low temperature flame photometer. Chlorides were measured by the electrometric method of

Best (1929) except that an arithmetical correction technique was applied in order to obtain greater accuracy in the analysis of the "0 NaCl" plants and the repeat extractions.

Three repeat extractions were done on the residues of high-salt mature leaves, and two on high-salt root samples, in order to check the effectiveness of the extraction procedure. For the mature leaves, chloride averaged 0.2, sodium 0.9, and potassium 2.1 per cent. of the values for the initial extractions. The corresponding values for the root samples were, chlorine 1.3, sodium 2.6, and potassium 3.0 per cent. These percentages were considered to be small enough to neglect, as they fell within the random errors involved in the plant culture and analytical methods used.

### III. EQUILIBRIUM EXPERIMENT

#### (a) Growth and Salt Tolerance

The dry matter yields of the roots and tops of the plants from the NaCl series show an almost linear decrease in growth for increasing external NaCl concentration (Plate 1; Fig. 1). The stimulating effect on growth of low external NaCl concentrations (Fig. 1) has been considered in more detail (Black 1956b, p.73).

A measure of the relative salt tolerance of *A. vesicaria* and *A. hastata* can be obtained by comparing the yields at various high external NaCl concentrations, as the yields of the two species at low external concentrations were closely comparable (Black 1956a). For instance, with *A. vesicaria*, top yields of 5 g and over occurred right up to the 0.7M culture level, whereas with *A. hastata* such yields ceased abruptly after the 0.3M culture level. *A. vesicaria* appears to have a greater tolerance of highly saline water cultures than *A. hastata*, in spite of the fact that only the latter species is an ecological halophyte.

#### (b) Analytical Results

(i) *Young Leaves*.—The sodium and chlorine levels in the young leaves of all plants lay very close to each other when plotted as equivalents (Fig. 2), although sodium was always present in slight excess except in treatments below 0.02M NaCl.

The relationship between internal and external NaCl concentrations was more linear when the internal NaCl levels were plotted on a leaf water or "cell sap" basis (Fig. 2(b)). This was because the *W/D* rose rapidly from a minimum of 3.42 (0 NaCl, series A) to a maximum of 7.87 (0.02M, series A), and thereafter fell to a minimum of 4.65 (1.0M, series A). All internal NaCl concentrations were greater than the corresponding external concentrations (Fig. 2(b)), and the concentration differences were greater at the lower end of the series.

(ii) *Mature Leaves*.—The chlorine equivalents on a dry matter basis for the mature leaves (Fig. 3(a)) were in general slightly lower than for the corresponding young leaves (Fig. 2(a)), the differences being greatest for the low-salt plants. This effect is similar to that already shown for *A. hastata* (Black 1956a). These differences for the high-salt plants of *A. vesicaria* were largely eliminated when the chlorine levels were plotted on a leaf water basis (Figs. 2(b), 3(b)) because, for these



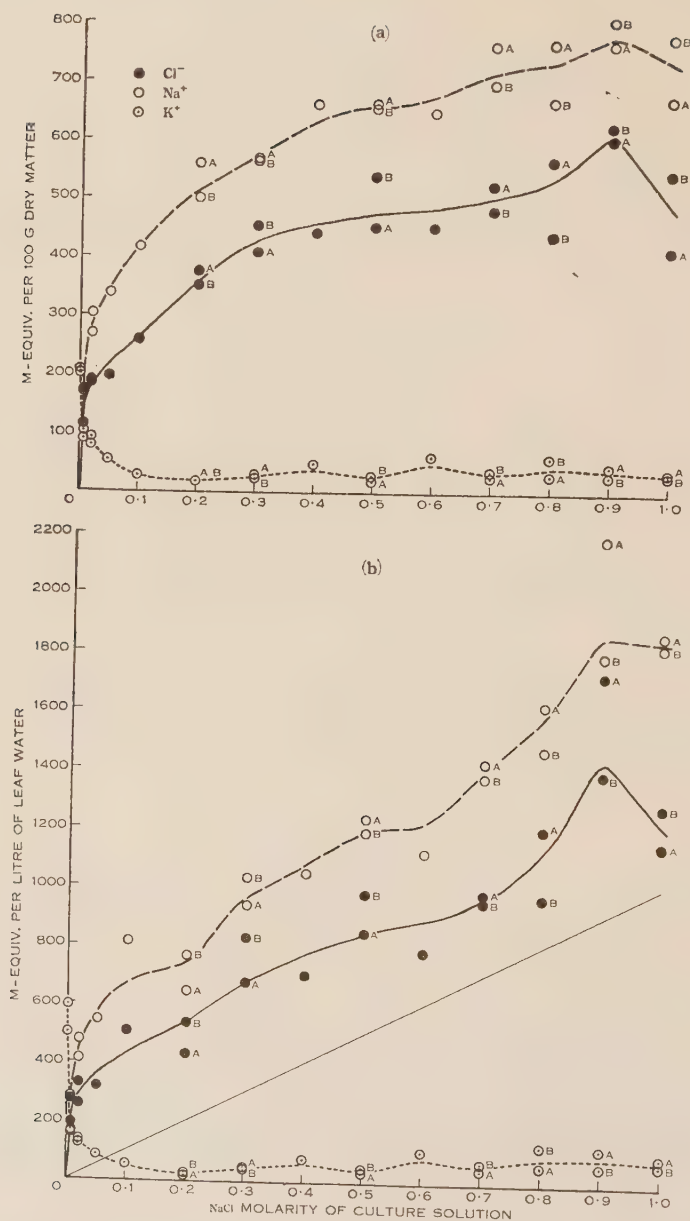


Fig. 3.—Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup> contents of mature leaves of *A. vesicaria* plotted against external NaCl concentration. Na<sup>+</sup> and Cl<sup>-</sup> contents for 0 NaCl treatments for the two series of plants respectively are (a) Na<sup>+</sup> = 6.8, 8.1; Cl<sup>-</sup> = 3.3, 1.8; (b) Na<sup>+</sup> = 20.0, 19.7; Cl<sup>-</sup> = 9.9, 4.3.

plants, the moisture contents of the young leaves were consistently higher than for the mature leaves.

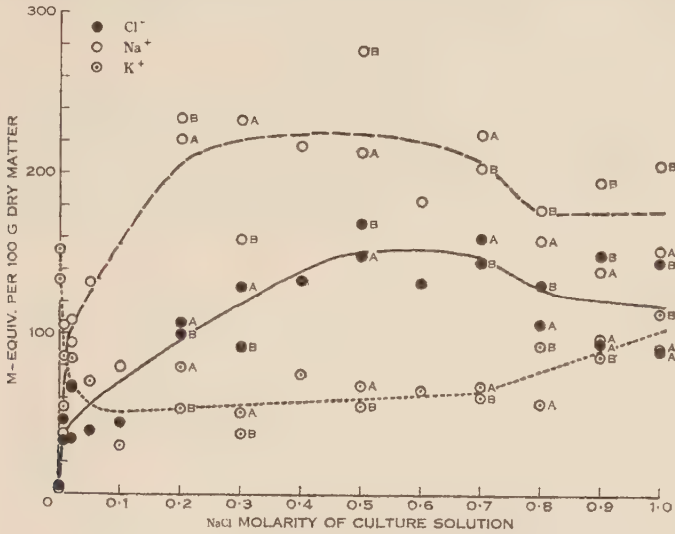


Fig. 4.— $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$  levels of young samples from root systems of *A. vesicaria* plotted against external  $\text{NaCl}$  concentration.

The sodium equivalents lay consistently above the chloride levels in the mature leaves (Fig. 3), and generally above the sodium levels in the corresponding

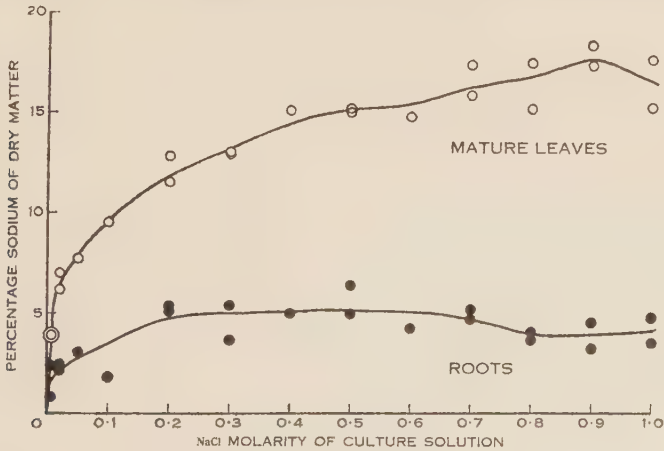


Fig. 5.—Comparative sodium levels of the mature leaves and roots of *A. vesicaria*. Percentage sodium contents for 0  $\text{NaCl}$  treatment for mature leaves = 0.16, 0.19; for roots = 0.07, 0.07 for the two series of plants respectively.

young leaves (Fig. 2). However, the potassium levels in the mature leaves were always below those of the young leaves with the notable exception of the 0  $\text{NaCl}$  treatment (Fig. 12).

The more linear relationship shown by plotting the internal  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations on a leaf water basis (Fig. 3(b)), were due to  $W/D$  trends similar to those noted for the young leaves; also internal ion concentrations were greater than the corresponding external concentrations.

(iii) *Roots*.—Here the sodium levels as equivalents were consistently above the chlorine levels, except in the 0 NaCl treatments (Fig. 4). This is a different situation to that shown for *A. hastata* (Black 1956a) where chlorine levels were dominant in at least the central portion of the range. There was a lowering of the internal sodium and chlorine levels for the three highest NaCl treatments (Fig. 4).

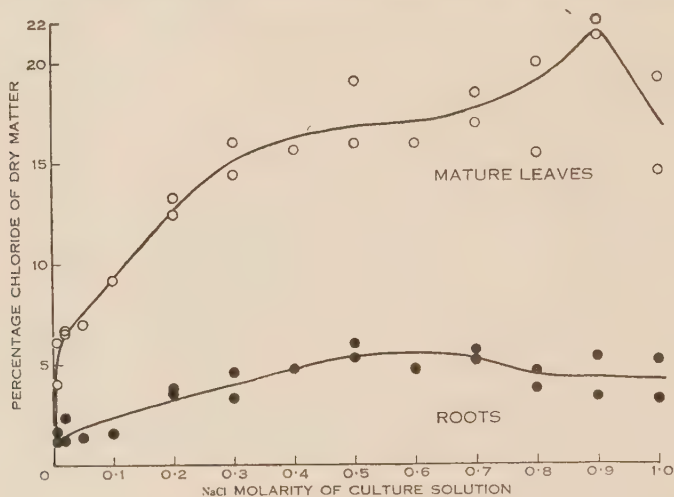


Fig. 6.—Comparative chlorine levels of the mature leaves and roots of *A. vesicaria*. Percentage chlorine contents for 0 NaCl treatment for mature leaves = 0.12, 0.06; for roots = 0.17, 0.18 for the two series of plants respectively.

Both the sodium and chlorine levels in the roots were considerably lower than the corresponding levels in the mature leaves (Figs. 5 and 6). This situation for chlorine was the exact opposite to that shown for *A. hastata* (Black 1956a). The differences tended to be greatest in the high-salt plants of *A. vesicaria* where the salt-uptake mechanism of the roots appeared to be more readily damaged by the high external NaCl concentrations.

#### IV. DYNAMIC ACCUMULATION EXPERIMENT

##### (a) *Young Leaves*

Over the sampling period, the sodium and chlorine levels of the young leaves rose rapidly (Figs. 7(a), 7(b), 7(d), 7(e)). This clearly indicated a high level of NaCl absorption as the final uptakes were close to those of the 0.1M treatment of the equilibrium NaCl series (Fig. 2).

The potassium levels were not significantly altered over the 10 days (Figs. 7(a), 7(d)) but the water relations effects were consistent and of interest. They

commenced as an immediate and significant drop in the  $W/D$  ratio which was most apparent in the samples collected 1 day after the transfer treatment; then followed a rapid recovery to about the original value from the third day on (Figs. 7(c), 7(f)).

(b) *Mature Leaves*

On a dry matter basis a slow absorption of NaCl was indicated over the sampling period, more particularly by the chlorine determinations (Figs. 8(a), 8(d)). The moisture content of the mature leaves dropped immediately and apparently

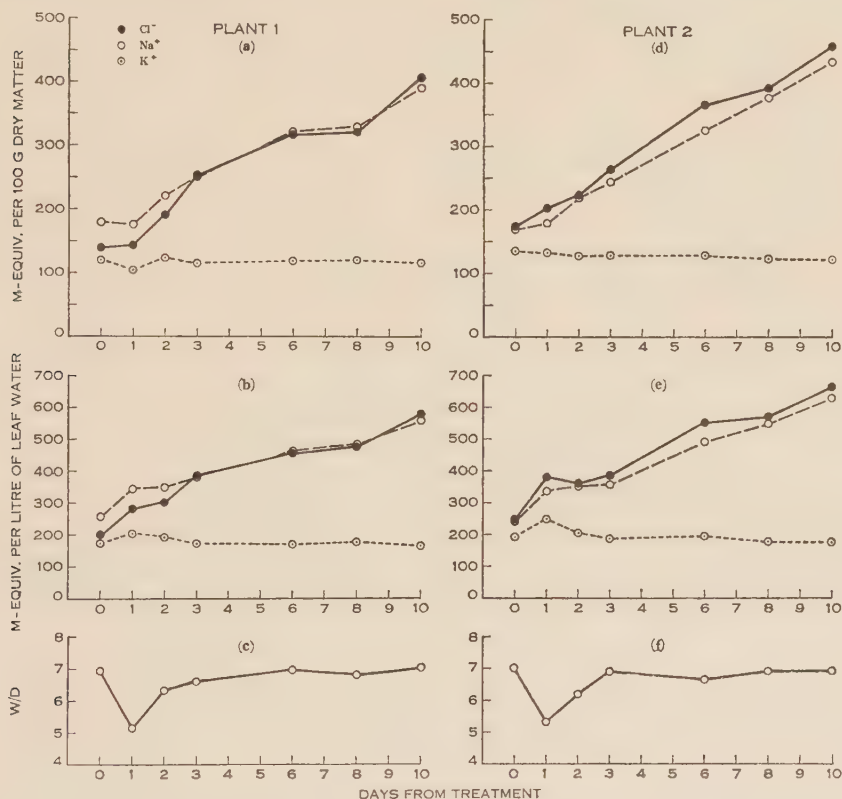


Fig. 7.—Salt accumulation in the young leaves of two *A. vesicaria* plants transferred at time 0 from a 0.006M to a 0.1M NaCl culture solution.

permanently (Figs. 8(c), 8(f)). This, of course, further increased the internal salt concentrations (Figs. 8(b), 8(e)). This mechanism was probably the main protection of the mature leaves against the suddenly increased external osmotic tension.

These results show that the salt-uptake mechanism of the mature leaves can do little more than maintain the high internal salt levels brought about in the developing leaves.



(c) *Roots*

The sodium and chlorine levels shown for the root systems over the sampling period (Figs. 9(a), 9(b)), probably did not change significantly, owing to the larger sampling errors involved in this material. Nevertheless, the results show that the

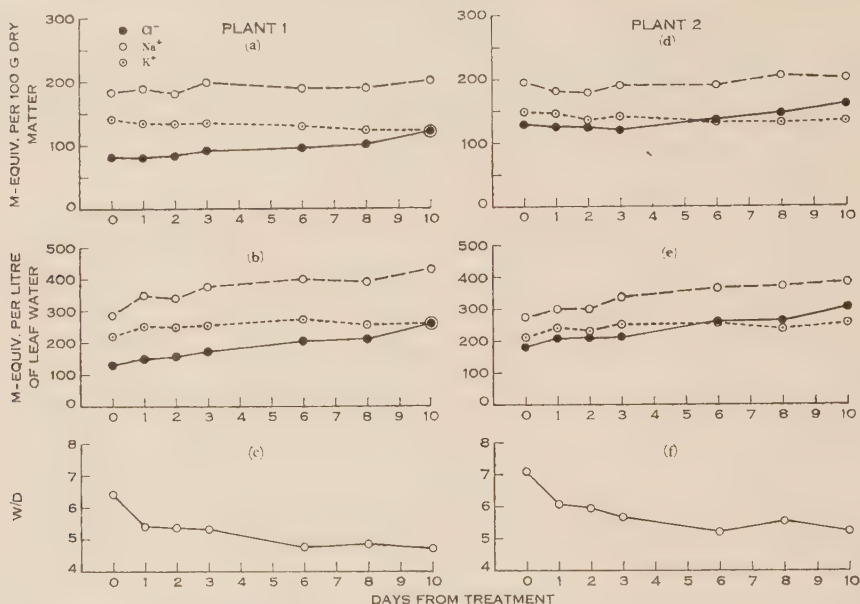


Fig. 8.—Salt changes in the mature leaves of two *A. vesicaria* plants transferred at time 0 from a 0.006M to a 0.1M NaCl culture solution.

NaCl contents did not greatly increase in the roots, although a rapid absorption rate must have been taking place to supply the demands of the young leaves (Fig. 7).

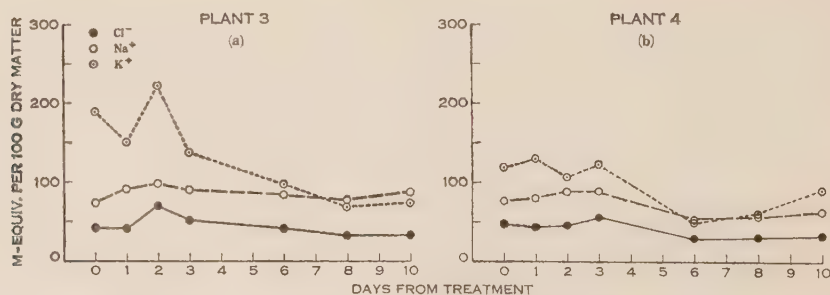


Fig. 9.—Salt changes in the roots of two *A. vesicaria* plants transferred at time 0 from a 0.006M to a 0.1M NaCl culture solution.

The NaCl absorption into the roots apparently had disturbing and depressing effects on the potassium contents (Figs. 9(a), 9(b)). This was the only occasion in this experiment, when mutual competition effects between Na<sup>+</sup> and K<sup>+</sup> appeared.

(d) *Individual Ions by Weight*

The plotting of the same results on a dry matter basis, and for the individual ions studied (Fig. 10), illustrated more clearly the different responses of the plant parts. The results for potassium (Figs. 10(a), 10(d)) showed that a marked depression of internal levels occurred only in the roots. However, the sodium results

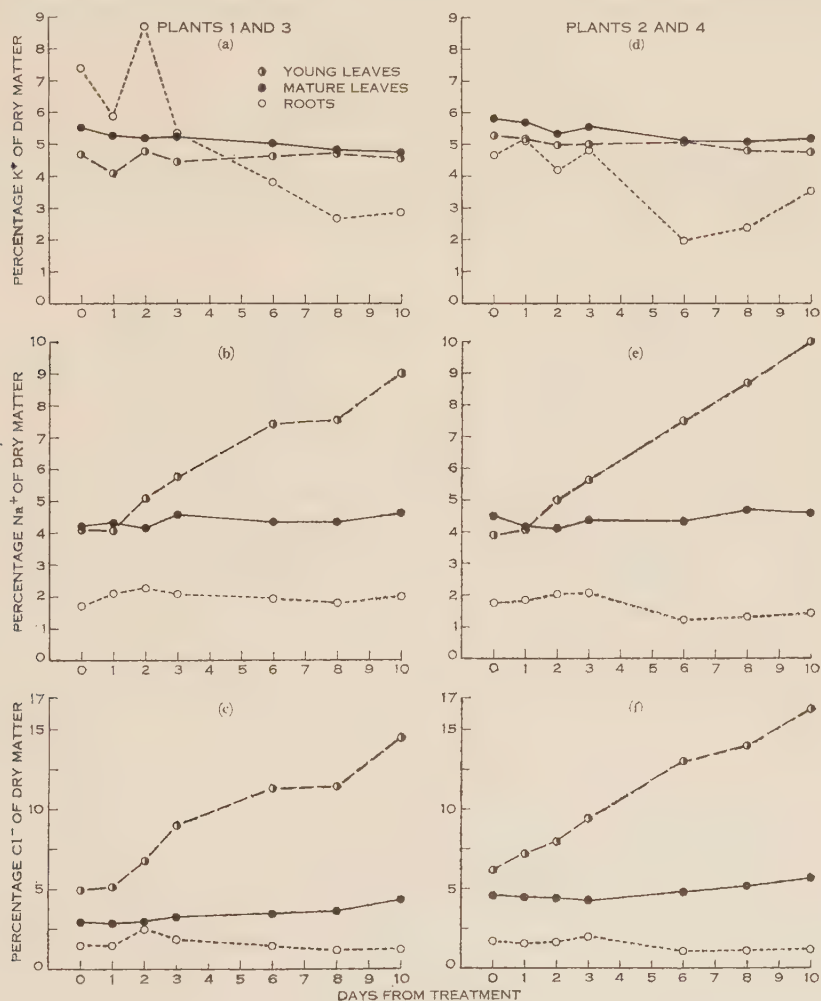


Fig. 10.—Responses of individual ions by weight for the young leaves, mature leaves, and roots of four *A. vesicaria* plants transferred at time 0 from a 0.006M to a 0.1M NaCl culture solution. Plants 1 and 2 were sampled for leaves, and plants 3 and 4 for roots.

(Figs. 10(b), 10(e)) showed that an increase of internal levels occurred only in the young leaves.

This indicated that the adsorbed Na<sup>+</sup> ions causing mutual competition and reduced potassium absorption at the root surfaces, were either insufficient to increase

root sodium levels, or were removed by the rinsing procedure at sampling. The reduction of root potassium levels after the third day would result from the movement of potassium to the new growth which amounted to about two nodes on each stem over the 10 days.

The chloride percentages (Figs. 10(c), 10(f)) indicated a rapid uptake of the ion into the young leaves and a much slower uptake into the mature leaves.

## V. GARDEN PLOT TRIAL

### (a) Plant Culture and Methods

The following is a brief summary of some results from a garden plot trial. These are given to form a comparison with the results obtained from water cultures, and to point out agreements and anomalies in the plant responses under a completely different set of conditions.

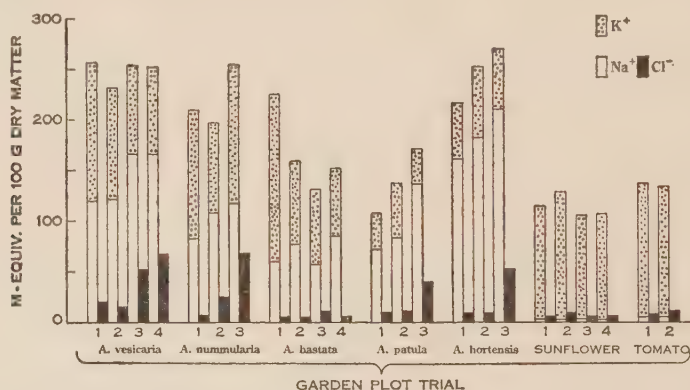


Fig. 11.—Cation and anion levels of Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup> for the mature leaves collected from various species grown in a garden soil.

Plants of *A. vesicaria*, *A. nummularia* Lindl., *A. hastata*, *A. patula* L., *A. hortensis* L., sunflower (cv. Russian Giant), and tomato (cv. Burwood Prize) were randomized in a plot running east and west behind the Botany School, Sydney. Seedlings were set out at times to allow for a full vegetative growth by a sampling date in January. No fertilizers were used but the plot was kept cultivated for weed control. The *A. hortensis*, sunflower, and tomato plants had all flowered by the sampling date while the other *Atriplex* species were still only in vegetative growth. Samples of mature leaves were collected and analysed using the same techniques as for the water-culture experiments.

The results indicated that the NaCl levels in the soils were higher towards the western end. The cation and anion levels were thus recorded with the plants being numbered from east to west (Fig. 11).

### (b) Results

The chloride results show that the two xerophytic *Atriplex* species, *A. vesicaria* and *A. nummularia*, were able in some cases to take up comparatively large concentrations of NaCl, although the highest of these (2.4 per cent. Cl of dry matter)

were about half the chlorine levels of the 0.006M NaCl culture solutions (Figs. 3(a), 6). These in turn were below the lower range of leaf chloride percentages (about 9 per cent. Cl of dry matter) for field specimens of *A. vesicaria* as determined by Wood (1925).

On the other hand, a lower absorption capacity was indicated for *A. hastata*, where the chloride levels were comparable to those for the sunflower and tomato plants. However, two higher chloride levels occurred in *A. patula* and *A. hortensis* plants; these species are ecologically and anatomically fairly close to *A. hastata*.

The cation-anion balances (Fig. 11) clearly illustrated the high selectivity of all the *Atriplex* species for the sodium ion. Nevertheless, potassium levels were usually comparable to those of the sunflower and tomato plants. Thus *Atriplex* species, generally, appear to have a higher capacity than most plants for absorbing monovalent cations.

## VI. DISCUSSION

### (a) NaCl Accumulation and the Donnan Equilibrium

Assuming salt concentrations expressed on a leaf water basis to be a measure of the mean cell sap concentrations, the results show that chloride concentrations of both young and mature leaves are approximately linearly proportional to the external concentrations, but exceeded the external concentrations by a more or less constant amount, which for the mature leaves was about 300 m-equiv/l (12 atm osmotic pressure) over the whole NaCl range (Fig. 3(b)).

Somewhat similar results have been described by Ashby and Beadle (1957) who found for two other xerophytic *Atriplex* species, *A. nummularia* and *A. inflata* F.Muell., that the cryoscopic osmotic pressures of expressed sap from their plus-salt plants were about 16 atm greater than the culture solutions. However, a different physiological response has been shown for *A. hastata* (Black 1956a) where mature leaf chloride concentrations were consistently below culture solution levels and Donnan effects appeared to dominate the equilibrium ion balance for culture solutions above 0.2M NaCl.

In the present equilibrium experiment, there is little indication of a Donnan effect in the young leaves (Fig. 2) because the total cation equivalents ( $\text{Na}^+$  and  $\text{K}^+$  plus other nutrient ions) is approximately equal to the chlorine equivalents. In the mature leaves (Fig. 3) the total cation is considerably in excess of chlorine equivalents indicating that the excess cations are held by a Donnan system, probably involving immobile organic anions.

Information about the processes involved in maintaining an approximately constant chlorine level in the leaves with time and maturation comes from the data for the dynamic experiment. In plants transferred from 0.006M to 0.1M NaCl, the salt content of the young leaves increased rapidly (Fig. 7) correcting the leaf water deficits caused by the osmotic pressure of the 0.1M solution. Apart from a very small increase in chlorine, the salt content of the mature leaves did not alter during the 10 days of the experiment (Fig. 8).

If it is assumed that the movement of salt from roots to leaves is via the xylem, then the rate of influx of ions into both young and old leaves should be



similar. Since the salt content of the old leaves does not increase, then either there is no influx of salt into mature leaves or the influx is balanced by an efflux possibly via the phloem. In field trials (Table 1) the rate of transpiration of old leaves was found to be similar to that of young leaves suggesting that an influx of salt into old leaves would occur. It is concluded that the influx in the young leaves greatly exceeds efflux and that as the leaves age, efflux gradually becomes more significant. The rapid retention of NaCl within the mesophyll tissues of the young leaves indicates a high capacity for accumulation.\*

TABLE 1  
RELATIVE TRANSPIRATION RATES OF YOUNG AND MATURE LEAVES OF A.  
VESICARIA\*

Water loss determined by weight of cut shoots from winter growth of field plants at Fowler's Gap, N.S.W. Trial carried out from 1 to 2 p.m. on an overcast day but with medium wind

Type of Leaf	Percentage Leaf Water Lost over First Hour (mean $\pm$ S.D. of 5 shoots)
Young leaves (terminal buds and first two pairs of nodes)	10.8 $\pm$ 0.8
Mature leaves (shoots 3-6 cm long after removal of tips)	12.3 $\pm$ 1.1

\* Same variety as used for water cultures.

No doubt these processes were also operative in the plants of the equilibrium series. The approximately constant NaCl concentration from young to mature leaves implies a rapid accumulation rate in the young growing leaf, but no net uptake once maturity was reached. The salt then must be leaving the mature leaf at a rate to balance the intake.

For the dynamic experiment, the relative rates of chlorine absorption and retention for the three organs studied were young leaves  $\gg$  mature leaves  $>$  roots (Figs. 10(c), 10(f)). For the absolute amounts of chlorine absorbed per organ, the relative uptake of young leaves would have been even further ahead because of appreciable growth (in the order of 100 per cent. increases) over the last 7 days of the sampling period.

### (b) Cation Selectivity

Mutual competition effects were shown by the plant sodium and potassium levels for the treatments above 0 NaCl (Figs. 2, 3, 4, 12), though the effects were complicated by near potassium depletion in the culture solutions supporting the biggest plants. These took up approximately 21 m-equiv. of potassium per plant from the initial total supply of 24 m-equiv.

\* The biological mechanism for concentrating both ions of a salt against a concentration gradient utilizing energy from metabolism. This is accumulation as defined by Robertson (1951).

As with *A. hastata* (Black 1956a), competition was most marked when potassium levels were plotted on a leaf water basis (Figs. 2(b), 3(b)). This was because for the two series, A and B, the  $W/D$  ratio of the young leaves rose from 3.4, 4.5 for the 0 NaCl treatments to 6.5, 5.9 for the 0.006M treatments, and the  $W/D$  ratio of the mature leaves rose from 3.4, 4.1 to 6.2, 5.9 respectively; some of the higher NaCl treatments produced even higher  $W/D$  ratios.

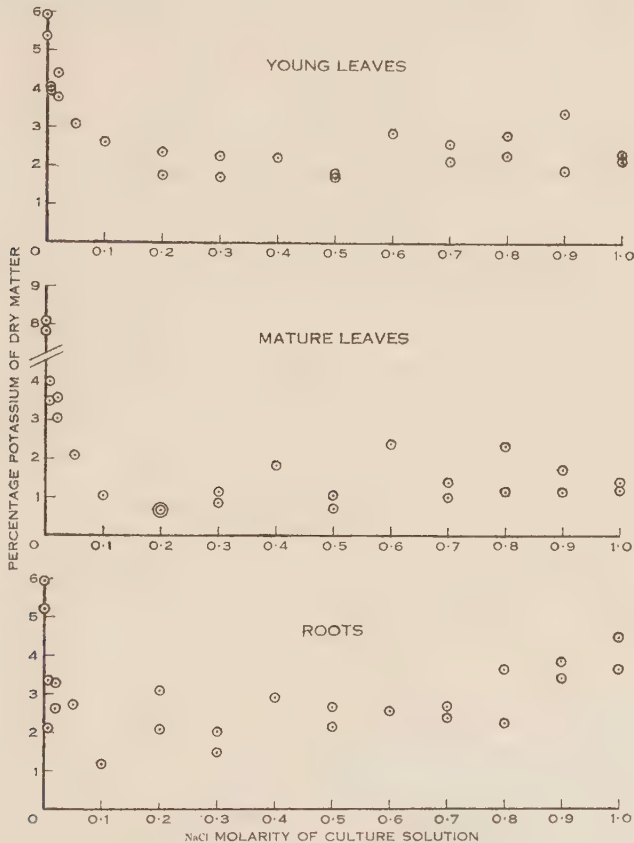


Fig. 12.—Potassium percentages of dry matter of *A. vesicaria* plants plotted against external NaCl concentration for young leaves, mature leaves, and roots.

The evidence for both *A. vesicaria* and *A. hastata* suggests that the uptake of potassium into these plants can best be considered as resulting from two different mechanisms. Firstly, there is a "luxury" uptake, where the potassium is completely exchangeable with sodium, and which is reduced to zero at high NaCl levels. Secondly, there is a specific uptake which, although it may vary somewhat according to potassium availability, is completely independent of the sodium-uptake mechanism. Bange (1959) has postulated a similar selective absorption of potassium and sodium for maize seedlings based on two distinct carrier systems.

The salt-accumulation process needed to maintain potassium levels in plants subjected to high NaCl treatments must require a highly specific active transport mechanism. Collander (1941), working with a number of species including *Atriplex*, was able to distinguish between the competitive properties of two groups of mono-valent cations:  $K^+$ ,  $Rb^+$ ,  $Cs^+$ , as distinct from  $Na^+$  and  $Li^+$ . Within the groups competition was complete; between the groups, only partial. Epstein and Hagen (1952), working with excised barley roots, studied similar effects and concluded the existence of several distinct binding sites of which one group binds  $K^+$ ,  $Rb^+$ , and  $Cs^+$  in preference to  $Na^+$  and  $Li^+$ .

TABLE 2

POTASSIUM AND SODIUM CONTENTS OF *A. VESICARIA* PLANTS GROWN IN CULTURE SOLUTIONS INITIALLY CONTAINING EQUIMOLAR CONCENTRATIONS (0.006M) OF POTASSIUM AND SODIUM

Values expressed as m-equiv/100 g dry matter

Plant Designation	Young Leaves		Mature Leaves		Roots	
	Potassium	Sodium	Potassium	Sodium	Potassium	Sodium
A	103	162	102	169	54	37
B	101	142	89	169	86	105
1	119	178	141	183	—	—
2	134	169	148	195	—	—
3	—	—	—	—	189	74
4	—	—	—	—	119	76
Mean K/Na ratio	0.70		0.67		1.53	

In studies of the cation-exchange capacities of cereal roots, Lundegårdh (1954) determined the following order of adsorption densities for the alkali elements on cation carriers. This was  $(H^+) > K^+ > Cs^+$  and  $Rb^+ > Li^+ > Na^+$ . From equimolar solutions (0.0025M) of chlorides he found an absorption ratio for K/Na of 24. He stated, "The slow entrance of Na is obviously caused by a low percentage of carriers suitable for this ion."

Collander (1941) determined similar K/Na ratios for glykophytes, when these were grown in culture solutions containing equimolar concentrations of potassium and sodium (0.004M). However, he showed that halophytes took up a great deal more sodium than these, although the K/Na ratio still remained greater than unity. Comparable results for *A. hastata* agreed with this finding (Black 1956a).

Potassium and sodium levels in six *A. vesicaria* plants grown in culture solutions initially containing equimolar concentrations (0.006M) of potassium and sodium are presented in Table 2. Culture treatments were exactly the same for all plants, but the reduced growth period allowed for the latter (plants 1-4) probably accounts for their generally somewhat higher ion uptake.

These results in Table 2 clearly show that the leaves of *A. vesicaria* absorb larger quantities of sodium than potassium from equimolar concentrations. The results for the root samples are less satisfactory, but nevertheless indicate remarkably low K/Na ratios. K/Na ratios of less than unity for the leaves indicate that *A. vesicaria*, a xerophyte, is more highly specialized with respect to sodium absorption than the halophytes that have been examined to date.\* Thus in the leaves of this species a majority of the alkali cation carriers must be eminently suitable for sodium absorption, even though a smaller proportion may be completely specific for potassium.

### (c) Conclusions

It is difficult to form conclusions on the functional value to *Atriplex* species of their high selectivity for the sodium ion even though it is now known to be an essential micronutrient for *A. vesicaria* (Brownell and Wood 1957). Growth responses to sodium salts at macro levels by members of the Chenopodiaceae are quite commonly recorded (e.g. Keller 1925; van Eijk 1939; Black 1956b; Ulrich and Ohki 1956; Ashby and Beadle 1957). The usual explanation is that sodium partially substitutes for potassium in metabolism and that this may benefit the chenopodious plant specially when potassium supply is deficient.

Ashby and Beadle took a different attitude to explain their 0.4 and 0.6M KCl treatments which were very toxic and led to extraordinary high luxury uptakes of nearly 30 per cent. potassium of dried leaf material. They claimed an antagonism function for sodium which they supposed would reduce these lethal potassium concentrations. Though perhaps true enough for these experimental conditions, a better interpretation of these results, and those of this paper, would be to recognize the specialized sodium uptake of the Chenopodiaceae as an essential part of an osmoregulatory mechanism, important to both halophytic and xerophytic members. In a low-sodium growth medium, the non-specific sodium carriers of the active transport mechanism would combine with K<sup>+</sup> ions and bring about a luxury uptake of potassium which could become toxic in a high-potassium medium. Thus, for these conditions, the excess potassium in the plant should be looked on as substituting for sodium, and the whole competitive interaction (as so well brought out by the osmotic pressure determinations of Ashby and Beadle) considered as osmoregulatory in character and not nutritional.

Heterogeneity of alkali cation carriers can also explain the apparent contradiction of higher potassium uptakes for high-potassium culture solutions than sodium for high-sodium solutions of these authors and the lower potassium uptakes of the equimolar culture solutions described here (Table 2). In the former case, all the alkali cation carriers would be available for potassium absorption, whereas against high-sodium culture solutions only the Na<sup>+</sup> carriers could function, the K<sup>+</sup> carriers being involved in maintaining nutritionally essential potassium levels. Only in equimolar solutions would a direct balance of absorption capacities for the two elements be involved.

\* Sutcliffe (1957) has obtained K/Na ratios of <1 for red beet root tissue under conditions conducive to high accumulation rates.



## VII. ACKNOWLEDGMENTS

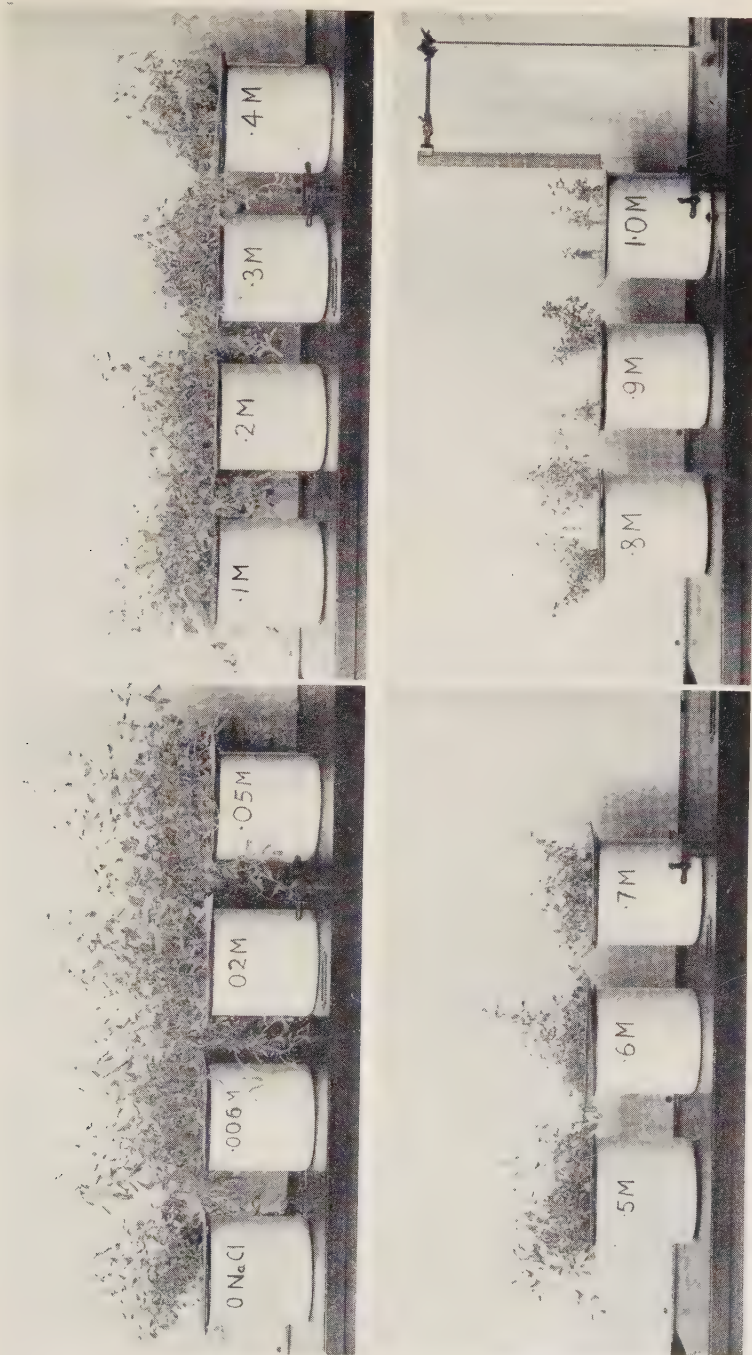
This paper represents part of the work submitted for the Ph.D. degree of the University of Sydney. Valuable early guidance and stimulation came from Professor N. C. W. Beadle, Botany Department, University of New England, Armidale. The later supervision and guidance is gratefully acknowledged of Professors R. L. Crocker and F. V. Mercer, Botany Department, University of Sydney, and Dr. R. N. Robertson, Division of Food Preservation and Transport, C.S.I.R.O.

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## ION UPTAKE AND GROWTH OF ATRIPLEX VESICARIA



An *A. vesicaria* series after 18 weeks of growth in water cultures.



# IONIC RELATIONS OF CELLS OF *CHARA AUSTRALIS*

## II. THE INDIFFUSIBLE ANIONS OF THE CELL WALL

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[Manuscript received February 19, 1960]

### Summary

Studies of isolated cell walls from *Chara australis* have been extended to measure the concentration of the endogenous anions of the wall and the  $pK$  of the acids which ionize to give these anions. The concentration of indiffusible anions in the wall is 0.8 equiv/l when the external concentration of cations is 20 mM but may be higher when it is greater than this. The mean  $pK$  of the acids from which the wall anions are derived is 2.2.

The number of indiffusible anions in wall segments of different thickness is proportional to thickness which indicates uniform addition of wall anions during growth and thickening.

The effect of some chemical treatments on the number of exchange sites in wall segments is consistent with their origin being carboxyl groups of polyuronic acids. Chemical analysis has subsequently shown that 15 per cent. of the dry weight of walls is uronic acid. This is mostly in the form of unmethylated polygalacturonic acid but some of the uronic acid is found associated with hemicellulose and cellulose.

## I. INTRODUCTION

In an earlier paper (Dainty and Hope 1959) evidence was given showing that most of the quickly exchangeable cations in internodal cells of *Chara australis* R. Br. var. *nobilis* A. Br. are to be found in the cell wall.

The free space of the wall was measured using radioactive iodide and mannitol and the cation exchange using  $^{22}\text{Na}$  and  $^{45}\text{Ca}$ . The kinetics of the exchanges of both cations and anions, and the amounts involved, strongly suggested that the cell wall was a complex system of indiffusible anions and could be considered as a Donnan free space (D.F.S.) and a water free space (W.F.S.). The exchange was complex in that the kinetics did not correspond to "single compartment exchange", indicating various degrees of accessibility of the exchange sites.

It is of great interest to try to identify the endogenous anions of the cell wall (the Donnan ions) with a particular chemical constituent.

The present paper is concerned with measurements of the concentration of the indiffusible anions and of the  $pK$  of the acids from which they are derived. Measurements have also been made of the number of exchange sites in walls of different physiological age and thickness, and finally, the effect of certain chemical treatments on the number of wall anions.

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## II. EXPERIMENTAL

Reference should be made to Dainty and Hope (1959) for details of the material used. In nearly all the present experiments, 1-cm segments of isolated cell walls were equilibrated in inactive solutions for several days followed by an equal period in a labelled solution of the same concentration. Then, after blotting, the wall segments were dried on standard planchettes and their radioactivity determined by direct counting. The specific activity of the solution with which the wall had been in equilibrium was then measured by counting an aliquot of the solution, usually 20  $\mu$ l, which was dried in the centre of the planchette, together with a "dummy" wall segment. Then, assuming equality of specific activity, the amount of a cation in a segment could be calculated from its radioactivity.

Usually three to five segments from different walls were measured and the mean taken.

TABLE 1

AMOUNTS OF EXCHANGEABLE SODIUM IN *CHARA AUSTRALIS* WALL SEGMENTS IN EQUILIBRIUM WITH EXTERNAL SOLUTIONS OF THE COMPOSITION SHOWN

Sodium Chloride Concn. (mM)	Calcium Chloride Concn. (mM)	Exchangeable Sodium ( $\mu$ -equiv/cm)	Sodium Chloride Concn. (mM)	Calcium Chloride Concn. (mM)	Exchangeable Sodium ( $\mu$ -equiv/cm)
5.00	0	0.080	4.70	0.30	0.028
4.97	0.03	0.065	4.00	1.00	0.016
4.90	0.10	0.041			

## III. RESULTS

*(a) Concentration of Indiffusible Anions in the Wall*

An average value for this concentration of 0.6 equiv/l of water in the D.F.S. was found by Dainty and Hope (1959) from the amount of exchangeable calcium divided by the apparent volume of water in the D.F.S. The latter was calculated from the difference between the iodide free space and the total water space in the wall (measured as mannitol free space). Since all these measurements are subject to experimental error, the above value for the indiffusible anion concentration in the D.F.S. is approximate and another determination by a different method was considered desirable.

In the following experiments the indiffusible anion concentration was calculated from the amount of exchangeable sodium (or calcium) in walls which had been equilibrated with solutions containing various mixtures of sodium chloride and calcium chloride. Wall segments were soaked for several days in renewed solutions of the composition shown in Tables 1 and 2. They were then blotted and transferred to a solution of the same total concentrations but labelled with  $^{22}\text{Na}$ .

After 2-3 days in the radioactive solution the segments were blotted, dried, and counted. Table 1 shows the amount of exchangeable sodium in a typical experiment. The amount of sodium in the W.F.S. is negligible in comparison with that in the D.F.S.

The progressive decrease in exchangeable sodium as external calcium concentration,  $[Ca_0]$ , is increased is qualitatively as expected from a Donnan system. Appendix I(a) gives the method used to calculate  $A$ , the effective concentration of the indiffusible anions, from such data. Table 2 summarizes the values of  $A$  so calculated for total (sodium+calcium) concentrations of 5, 10, and 20 mN. The approximate constancy of  $A$  at a given total external concentration is consistent with our assumption of a Donnan equilibrium. The fact that  $A$  is less when the total external concentration is 5 mN than when it is 10 or 20 mN could be due to

TABLE 2

VALUES FOR THE APPARENT CONCENTRATION  $A$  OF INDIFFUSIBLE ANIONS IN CHARA AUSTRALIS CELL WALLS WHICH ARE IN EQUILIBRIUM WITH EXTERNAL SOLUTIONS OF THE COMPOSITION SHOWN

Sodium Chloride Concn. (mN)	Calcium Chloride Concn. (mN)	$A$ (equiv/l)	Sodium Chloride Concn. (mN)	Calcium Chloride Concn. (mN)	$A$ (equiv/l)	Sodium Chloride Concn. (mN)	Calcium Chloride Concn. (mN)	$A$ (equiv/l)
4.97	0.03	0.45	9.9	0.1	0.74	19.9	0.1	0.96
4.90	0.10	0.41	9.7	0.3	0.88	19	1	0.74
4.70	0.30	0.36	9.0	1.0	0.76	18	2	0.61
4.00	1.00	0.30	7.0	3.0	0.79	15	5	0.69

several causes which will be considered in Section IV. The values of  $A$  at 10 or 20 mN are not significantly different from each other and the mean value of  $A$  at either of these two concentrations can be taken as 0.8 equiv/l. There are indications that the apparent  $A$  is greater at external concentrations greater than 20 mN (Dainty and Hope, unpublished data).

#### (b) *pK of the Cell Wall Anions*

Wall segments were equilibrated in solutions of sodium chloride brought to pH values between 2.2 and 8 by addition of McIlvaine's buffer (citric acid + disodium hydrogen phosphate). The total concentration of sodium was kept at 20 mN in each solution. The segments were then transferred to labelled buffered solutions of the same chemical composition (labelled with  $^{22}Na$ ) and, after sufficient time to come to equilibrium (2-3 days), were blotted, dried, and the radioactivity

counted as before. Figure 1 gives the total exchangeable sodium (in  $\mu\text{-equiv/cm}$  of wall) as a function of the pH of the buffered solution. The relation is consistent with a decreased ionization of the indiffusible anions as the external pH is decreased (i.e. the acidity increased) such that half-maximum ionization occurs at an external pH of about 3.7. The amount of the sodium in the W.F.S. in these experiments was less than  $0.01 \mu\text{-equiv/cm}$  and can be neglected in comparison with that in the D.F.S.

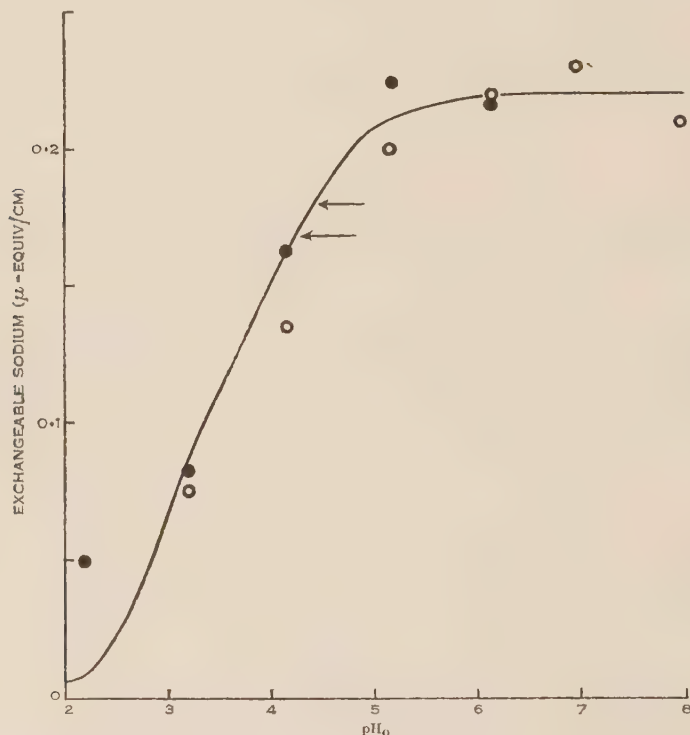


Fig. 1.—Amount of exchangeable sodium (in  $\mu\text{-equiv/cm}$  of wall) plotted against the pH of buffer solutions, each containing 20mM sodium (two experiments). The exchangeable sodium in wall segments in unbuffered 20 mM NaCl is indicated by the arrows. The line is the theoretical relation according to Appendix I(b), for a  $pK$  of 2.2 and a maximum concentration ( $a$ ) of indiffusible anions equal to  $0.8 \text{ equiv/l.}$

In Appendix I(b) a theoretical analysis is given of the amount of exchangeable sodium as a function of external pH ( $pH_0$ ) on the assumption that all the anionic groups have, effectively, the same  $pK$  value. The curve shown in Figure 1 is the relation between the internal sodium concentration,  $[Na_i]$ , and external pH assuming an indiffusible anion concentration (in the fully ionized state) of  $0.8 \text{ equiv/l.}$ , a  $pK$  of 2.2, and an external sodium concentration,  $[Na_o]$ , of 20 mM. Although this line is adjusted to give the same point of half-maximum ionization as the

experimental value, it is not a good fit at low pH. This could indicate that the wall contains a complex mixture of acids with different  $pK$  values.

(c) *Amount of Indiffusible Anions in Walls of Different Thickness*

The amounts of exchangeable calcium were measured in walls isolated from a strand of internodal cells of which the length and wall thickness increased progressively with distance from the apex. The thickness was calculated from the blotted weight, the density, and the area. The blotted weight and area were measured and the mean density of the walls was taken as  $1.1 \text{ g/cm}^3$ .

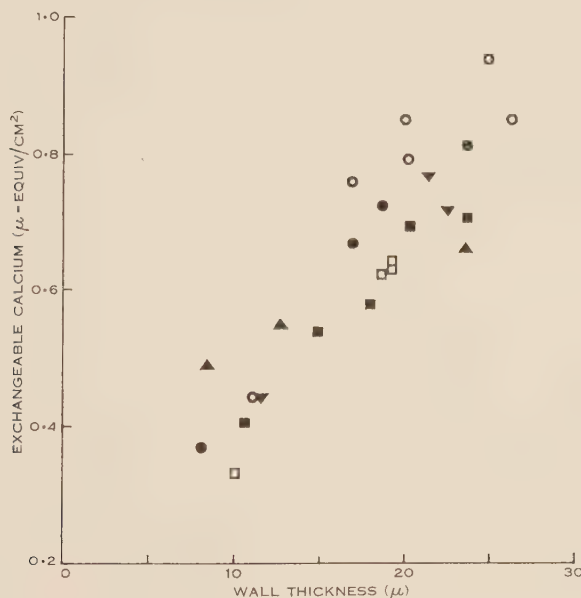


Fig. 2.—Amount of exchangeable calcium per unit area (in  $\mu$ -equiv/cm<sup>2</sup>) plotted against thickness of wall segments ( $\mu$ ). Each symbol represents a strand of cells from which the walls were isolated. In nearly all cases increase in wall thickness followed increase in cell length along the strand (from growing point downwards).

Figure 2 shows the exchangeable calcium per unit area plotted against the thickness of the wall. This gives a good measure of the number of indiffusible anions provided ionization is complete which is approximately true for  $[\text{Ca}_o] = 1 \text{ mM}$  and  $pK \leq 2.8$ . There is an approximately linear relation between number of endogenous anions per unit area and thickness, which suggests that as a young cell elongates and thickens the new wall material laid down has the same composition as the older wall, as far as concentration of fixed anionic groups is concerned.



*(d) Effect of Chemical Treatments on the Number of Wall Anions*

The number of wall anions, as indicated by the total amount of exchangeable calcium, was measured in untreated wall segments 1 cm long and also in segments which had been given the following treatments:

- (1) Boiled in distilled water for 12 hr. This treatment was designed to remove pectins of short chain length (Kertesz 1951).
- (2) Segments treated with 0.05N HCl at 70°C for 1 hr then with 0.5 per cent ammonium oxalate at 70°C for 1 hr. This is a recognized procedure for dissolving protopectins and pectates (Ordin, Cleland, and Bonner 1955).
- (3) Segments treated with 0.25 per cent. oxalic acid and 0.25 per cent. ammonium oxalate at 70°C for 1 hr. This treatment is supposedly more specific with regard to removing pectinic materials (Bishop, Bayley, and Setterfield 1958).
- (4) Treatment (2) followed by 17.5 per cent. sodium hydroxide at 25°C for 4 hr. This treatment leaves only  $\alpha$ -cellulose undissolved (Ordin, Cleland, and Bonner 1955), removing hemicelluloses as well as pectins.

TABLE 3

EFFECTS OF VARIOUS TREATMENTS ON THE TOTAL EXCHANGEABLE CALCIUM IN  
CHARA AUSTRALIS CELL WALLS

Results are expressed as percentage of exchangeable calcium remaining  
(untreated walls = 100 per cent.)

Treatment*	Exchangeable Calcium (% of untreated)	No. of Observations
Untreated	100	—
(1) Boiling water	92 $\pm$ 2.5†	6
(2) HCl-ammonium oxalate	8.9 $\pm$ 0.9	6
(3) Oxalic acid-ammonium oxalate	70.0 $\pm$ 0.5	3
(4) HCl-ammonium oxalate-NaOH	4.4 $\pm$ 1.2	6

\* See p. 272 for further details.

† Standard error of the mean.

After these treatments the walls were rinsed and placed in calcium chloride solution for 2-3 days and then into labelled calcium chloride solution for the same time.

Table 3 gives the results of several such experiments. Treatment (1) had little effect on the amount of exchangeable calcium while treatment (2) removed more than 90 per cent. of the exchange sites with a 27 per cent. change in dry weight. Treatment (4) had a further small effect. Treatment (3) removed 30 per cent. of the exchange capacity of the walls. If treatment (3) is more specific than (2) in bringing pectinic materials into solution, it might be concluded from the

above experiments that the indiffusible anions of the wall are associated with long-chain protopectin materials (since treatment (1) was almost ineffective), and also with other hexose materials removed by the less specific treatment (2).

Any protein from cytoplasm remaining in the interstices of the wall would presumably have been denatured rather than removed by treatments (1), (2), and (3). Nevertheless treatment (2) removed 90 per cent. of the exchange capacity. In any case cytoplasm contributes very little to the quickly exchangeable cations (Dainty and Hope 1959; Diamond and Solomon 1959).

In addition to the measurements of exchange capacity after the above chemical treatments, control walls and wall material after treatment (2) were compared in respect of the amount of carbon dioxide evolved under mild hydrolysis (12 per cent. HCl at 130°C for 2 hr (Kertesz 1951, p. 36)). Untreated material produced carbon dioxide equivalent to 2  $\mu$ -equiv/mg dry wt., and walls after treatment (2) evolved 0.1  $\mu$ -equiv/mg dry wt. A further slow evolution of carbon dioxide occurred after the initial 2 hr, probably due to breakdown of hexoses (Kertesz 1951). The equivalent amounts of carbon dioxide, before and after treatment (2), are reasonably close to the exchange capacities of the walls measured directly, i.e. 1.3  $\mu$ -equiv/mg dry wt. for untreated walls and 0.12  $\mu$ -equiv/mg dry wt. after treatment (2). It is thus possible to conclude that the exchange capacity is derived from  $\text{-COO}^-$  groups in the wall.

#### IV. DISCUSSION

It is clear from the measurements of the amounts of exchangeable sodium in the cell wall as a function of the concentrations of sodium and calcium in the external solution that, at total external concentrations of 10–20 mN, the concentration  $A$  of indiffusible anions in the D.F.S. of the cell wall is about 0.8 equiv/l. This agrees quite well, considering the possible experimental errors, with the previous value of 0.6 equiv/l (Dainty and Hope 1959), obtained by a different method. Thus the analysis of the cell wall into a D.F.S. and a W.F.S. is confirmed, since the *average* concentration of indiffusible anions in the cell wall is about 0.3 equiv/l of total wall water.

With an external concentration of 5 mN,  $A$  was about 0.4 equiv/l. Several factors could give rise to the discrepancy between this and the value of 0.8 given above. The acids may not be completely ionized at an external salt concentration of 5 mN, and replacement of calcium by sodium, which is very slow (Dainty and Hope 1959), may not have proceeded to equilibrium; the latter is the more likely explanation since if as little as 0.02  $\mu$ -equiv. calcium per centimetre of wall was present in segments "equilibrated" in pure sodium chloride (5 mN), it can be shown that  $A$  would be raised to 0.65 equiv/l.

The chemical treatments (Table 3) and analysis suggest that the indiffusible anions of the cell wall are carboxyl groups of polyuronic acids, since the standard treatments to solubilize these acids resulted in the loss of about 90 per cent. of the cell wall exchange capacity. Further, the equivalent of the carbon dioxide evolved under mild hydrolysis (the standard method of estimation of uronic acid carboxyl groups) corresponded approximately to the observed exchange capacity

both in untreated and acid-oxalate-treated walls. Since this work was completed Anderson and King (personal communication) have kindly made a more detailed analysis of *C. australis* wall material supplied by the authors. Fifteen per cent. of the dry weight was uronic acid. This corresponds to  $0.8 \mu\text{-equiv/mg}$  dry wt., assuming an equivalent weight of 194. A reasonable correction for contamination by cytoplasm adhering to the wall preparation would raise this value by up to 40 per cent. Thus there is good quantitative agreement between the average concentration of uronic acids and the average concentration of indiffusible anions (measured by our ion-exchange studies) in the cell wall. The chemical analyses also indicate the complex nature of the uronic acids; they may be part of a pure galacturonic acid chain (pectin) or interpolated with sugar residues in a copolymer (hemicellulose); and a small number will be present in the so-called cellulose chains. None of the uronic acid groups are methylated.

The carboxyl groups may well be in different regions of accessibility and cause complex exchange kinetics (cf. Dainty and Hope 1959). This complexity is also suggested in the "titration" curve of Figure 1, which is not fitted altogether satisfactorily by a single  $pK$  value. However, the mean  $pK$  of 2.2 is reasonably close to the minimum quoted for pectic acid (2.8), considering the experimental errors.

The results shown in Figure 2 indicate that the cell wall of *C. australis* is built up uniformly in the sense that the uronic acid concentration remains constant throughout growth and thickening of the wall.

This work, then, supports the conclusions of the earlier paper (Dainty and Hope 1959) and underlines the complexity of the cell wall system. The indiffusible anions are predominantly ionized uronic acid carboxyl groups, some on polygalacturonic acids, some mixed with sugars in copolymers, and a few on the cellulose chains. Thus some of the groups will be close together and others relatively isolated.

One theoretical concept should be mentioned which will be published elsewhere. The terms D.F.S. and W.F.S. have quantitative meaning only at a single external solution concentration and depend on what kind of ions are in the external solution. For this reason the term "apparent free space" was first used by G. E. Briggs (unpublished data). The size of the D.F.S. is related to the width of the electric double layers near the fixed charges. This depends, in turn, on solution concentrations and to some extent on the valency of the ions. This is why it is stated that the D.F.S. concentration is  $0.8 \text{ equiv/l}$  at external solution concentrations of 10–20 mM. Only when the electric double layers overlap to a considerable extent can the D.F.S. be considered a homogeneous phase and only then is the use of classical Donnan considerations permissible.

#### V. ACKNOWLEDGMENTS

Thanks are due to Dr. J. R. Vickery, Chief, Division of Food Preservation and Transport, C.S.I.R.O., and Professor R. L. Crocker, Botany School, University of Sydney, in whose Laboratories the work was carried out.

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## APPENDIX I

 (a) Calculation of the Indiffusible Anion Concentration,  $A$ 

From data on the change in exchangeable sodium as the  $[Ca_o]/[Na_o]$  ratio is changed we have, in the D.F.S., the Donnan distribution equation

$$\frac{Ca_i/v}{[Ca_o]} = \left( \frac{Na_i/v}{[Na_o]} \right)^2, \quad \dots\dots\dots (1)$$

where square-bracketed symbols represent concentrations and unbracketed symbols amounts, and  $v$  is the volume of the D.F.S. For consistency with earlier publications  $A$  is unbracketed.

Since the mobile anion concentration in the D.F.S. is negligible, then

$$A = [Ca_i] + [Na_i], \quad \dots\dots\dots (2)$$

the concentrations being in equiv/l. Eliminating  $v$  it can be shown that

$$A = (Ca_i + Na_i).Ca_i.[Na_o]^2/(Na_i^2.[Ca_o]). \quad \dots\dots\dots (3)$$

Since  $[Na_o]$  and  $[Ca_o]$  are known and  $Ca_i$  and  $Na_i$  are measured (i.e.  $Na_i$  directly,  $(Ca_i + Na_i)$  is taken as equal to  $Na_i$  when  $[Ca_o] = 0$ , and hence  $Ca_i$  by difference),  $A$  may be calculated. The sodium in the W.F.S. of the wall is negligible in comparison with that in the D.F.S.

## (b) Calculation of Exchangeable Sodium as a Function of External pH in Buffer Solutions

The following equations enable  $[Na_i]$ , the concentration of sodium in the D.F.S., to be calculated in terms of known quantities:

$$[Na_i]/[Na_o] = [H_i]/[H_o], \quad \dots\dots\dots (4)$$

$$A = [H_i] + [Na_i], \quad \dots\dots\dots (5)$$

$$A + [HA] = a, \quad \dots\dots\dots (6)$$

$$[H_i].A/[HA] = k. \quad \dots\dots\dots (7)$$

Equation (4) is the Donnan distribution equation, (5) expresses electrical neutrality in the D.F.S. (mobile anions can be neglected), (6) states that the maximum indiffusible anion concentration is  $a$ , while (7) expresses the ionization of the weak acid  $HA$  into  $H_i^+$  and  $A^-$  with dissociation constant  $k$ .



It can be shown that

$$[\text{Na}_i] = \frac{1}{2} \left\{ \left[ \frac{k^2[\text{Na}_o]^2}{[\text{H}_o]^2} + \frac{4k\alpha[\text{Na}_o]^2}{[\text{H}_o]( [\text{H}_o] + [\text{Na}_o] )} \right]^{\frac{1}{2}} - \frac{k[\text{Na}_o]}{[\text{H}_o]} \right\} \dots (8)$$

In Figure 1 the curve is the relation between  $[\text{Na}_i]$  and  $\text{pH}_o$ , assuming  $\alpha = 0.8$  equiv/l (Section II(a)),  $k = 6.3 \times 10^{-3}$  ( $\text{pK} = 2.2$ ), and  $[\text{Na}_o] = 20 \text{ mM}$  (constant).

# IONIC RELATIONS OF CELLS OF *CHARA AUSTRALIS*

## III. VACUOLAR FLUXES OF SODIUM

By A. B. HOPE\* and N. A. WALKER†

[Manuscript received February 19, 1960]

### Summary

Estimates have been made of the vacuolar influx ( $\phi_i$ ) and efflux ( $\phi_o$ ) of sodium in cells of *Chara australis* R.Br. var. *nobilis* A.Br. from measurements of radioactivity in extracted sap samples or from direct counts of single cells, using the isotope  $^{22}\text{Na}$ .

The influx was often less than the efflux, both being in the range 0.1–0.4 p-equiv/cm<sup>2</sup>. sec (1 p-equiv.  $\equiv 10^{-12}$  equiv.), at room temperature and in the light. Dark conditions reduced  $\phi_i$  and  $\phi_o$  to about 70 per cent. of their values in light. The  $Q_{10}$  of  $\phi_i$  and  $\phi_o$  was about 2.5.  $\phi_i$  was independent of external concentration in the range 0.2–2.0 mN but increased sharply when the sodium concentration was 5 mN. The sodium ion was shown to contribute less than 5 per cent. to an electric current passed through the cell surface and along the vacuole.

The vacuolar sodium concentration was very much less than its calculated value assuming electrochemical equilibrium with the external medium. The potassium in the vacuole was in approximate electrochemical equilibrium with that in the medium.

These facts are discussed in relation to possible mechanisms of sodium movement in *C. australis* cells.

## I. INTRODUCTION

Three separate ion-exchange processes occur between giant internodal cells of the Characeae and the external medium. The initial exchange was shown by Diamond and Solomon (1959) using *Nitella* cells and, independently, by Dainty and Hope (1959) using *Chara australis* cells, to take place in the cell wall. In *C. australis* indiffusible anions are present in the cell wall to the extent of about 2  $\mu$ -equiv/mg dry wt. They are thought to be derived from the ionization of polyuronic acids (Dainty, Hope, and Denby 1960). The second exchange, with a half-time of the order of 1 hr, is with the "protoplasmic non-free space" (N.F.S.) (MacRobbie and Dainty (1958). The amount of sodium in the N.F.S. of *C. australis* appears to be very small and this exchange is difficult to separate from that in the cell wall, which contains some counterions which are only slowly exchangeable. For this reason few measurements have been made of ions in the N.F.S. of *C. australis*.

The third and slowest exchange is with ions in the vacuole. Some measurements of vacuolar exchange have been made by Gaffey and Mullins (1958), MacRobbie and Dainty (1958), and Diamond and Solomon (1959). The fluxes range from 0.2 to 6 p-equiv/cm<sup>2</sup>. sec,‡ but it is likely that the apparently high values

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‡ 1 p-equiv.  $\equiv 10^{-12}$  equiv.

of Gaffey and Mullins were due to the presence of cortical cells in the species of *Chara* they used. MacRobbie and Dainty considered the vacuolar fluxes of sodium and potassium to be passive. The present paper is a more detailed study of the vacuolar influx and efflux of sodium under various conditions, using  $^{22}\text{Na}$  as a tracer.  $^{42}\text{K}$  will soon be available in Australia at high enough specific activity to be used in such experiments.

On the basis of the observed potassium, sodium, and chloride vacuolar fluxes, and assuming all except the chloride influx to be passive, MacRobbie and Dainty (1958) calculated the resistance of the tonoplast to be about  $250\text{ k}\Omega\text{ cm}^2$ . However, Walker (1957) found the outer membrane to have a resistance to the flow of electric current as low as  $6\text{ k}\Omega\text{ cm}^2$ , and the tonoplast to have a much lower resistance. In the present paper data comparing the vacuolar fluxes of sodium with those due to flowing electric currents are brought to bear on this discrepancy.

## II. EXPERIMENTAL METHODS

### (a) *Material*

Strands of *C. australis* R. Br. var. *nobilis* A.Br. were collected from field ponds and kept in an artificial pond water (A.P.W.) containing  $0.5\text{ mN CaCl}_2$ ,  $1.0\text{ mN NaCl}$ , and  $0.1\text{ mN KCl}$ . Individual internodal cells 3–7 cm long and 1–1.5 mm in diameter were cut from the strands and kept in A.P.W. for a period of at least 3 days. The A.P.W. was renewed about twice daily.

### (b) *Influx*

A cell of measured surface area was placed for 4–6 hr in A.P.W. with  $^{22}\text{Na}$  added to give a specific activity of 5–10  $\mu\text{C}/\mu\text{-equiv. Na}$ . After rinsing in inactive A.P.W. for about 1 hr to remove most of the cell wall  $\text{Na}^*$  (i.e.  $^{22}\text{Na}$ ), the cell was blotted lightly, one end cut off, and a sap sample taken. The sap was free from chloroplasts; contamination by colourless cytoplasm was unlikely as the sap was ejected by turgor pressure from the cut cell. The sample of vacuolar sap of known volume, 5–30  $\mu\text{l}$ , was diluted to 10 ml and placed in an annular container for scintillation counting using a sodium iodide crystal. The radioactivity of the vacuolar sap was compared with that of a small sample of labelled A.P.W. made up to 10 ml.

In a second method, the cell was counted directly at intervals by placing it in a small tube let into a slab of scintillating plastic.† This method relies for accuracy on placing the cell always in the same position and also on removing the wall  $\text{Na}^*$  by rinsing for about 20 min with inactive A.P.W. After this time a certain amount of radioactive sodium remains in the wall (Dainty and Hope 1959, particularly Figures 3 and 4) and possibly in the N.F.S. This is shown in some of the graphs below of counting rate against time, where the line extrapolated to zero time passes through the axis at 10–30 counts/sec. The amount of activity in the wall after rinsing for 20 min is probably approximately constant, being dependent on time of rinsing rather than accumulated time in  $\text{Na}^*$ , because of the comparatively rapid

† NE 102 plastic from Nuclear Enterprises Ltd.

exchange in the wall. In any case this method gave substantially the same answer for the influx as the direct sap count. In two experiments where the two methods were applied to the same cell, the influxes were: (1) 0.39 p-equiv/cm<sup>2</sup>. sec (whole cell count) and 0.35 (sap count); (2) 0.19 (whole cell count) and 0.25 (sap count). The cell activity was compared with that of a sample of labelled A.P.W. sealed into a glass capillary of about the same dimensions as the cell, to enable calculation of the influx in equivalents. Since only the  $\gamma$ -rays from <sup>22</sup>Na were counted, absorption corrections were unnecessary.

### (c) *Efflux*

The efflux of sodium was calculated from the radioactivity lost per unit time (usually 1 hr) from unit cell surface area of cells previously soaked for up to 3 weeks in labelled A.P.W. Na\* must be completely removed from the wall and cytoplasm before efflux from the vacuole can be measured. The cells were repeatedly rinsed in A.P.W. over a period of 48 hr. This removes Na\* from the walls of internodal cells (Dainty and Hope 1959). Nodal cells, with a much higher surface/volume ratio, will also lose their Na\* in this time if their membranes are similar to those of internodal cells in permeability.

After these repeated rinses, a single cell was placed in successive 10-ml aliquots of A.P.W. for periods of 1 hr and the radioactivity of each aliquot determined in the scintillation counter. Thus, effluxes were initially expressed in counts per min per hr. They were converted to p-equiv/cm<sup>2</sup>. sec after measurement of the specific activity of the sap at the end of the experiment. The vacuolar specific activity was almost constant over this period since of a total of 10<sup>5</sup>–10<sup>6</sup> counts/min, only 10<sup>3</sup>–10<sup>4</sup> were lost during the efflux measurements.

Counting rates of 50–300 counts/min for each hourly sample were commonly obtained in these experiments, so that the external specific activity never rose enough to limit the efflux. Calculation of the efflux involves knowing the vacuolar sodium concentration as well as its radioactivity. Owing to the danger and inconvenience of using radioactive samples for flame photometry, a number of inactive cells of the same batch as the experimental ones was analysed for vacuolar sodium (and potassium) concentration ([Na<sub>v</sub>], [K<sub>v</sub>]) and the mean taken.

### (d) *Variability of Fluxes*

The effect of various treatments on  $\phi_i$  and  $\phi_o$  is described below in Section III. A large number of experiments was done in which a set of 5–10 control cells was compared with an equal number of other cells under a different condition (e.g. of light or temperature). While these experiments demonstrated the gross effect, in some cases with high statistical significance, experiments were preferred in which a single cell could be given different treatments in successive time intervals. This eliminated the effect of variability amongst cells, which, together with seasonal effects, is the cause of the large standard errors in the means of  $\phi_i$  and  $\phi_o$  quoted below. It is such experiments with single cells which are used below to illustrate the results, together with some tables which compare the mean sap activity of sets of cells given different treatments.



## III. RESULTS

(a) *Mean Vacuolar Fluxes of Sodium in the Light at 20–27°C*

Averaged over 117 cells, the mean sodium influx from A.P.W. ( $\phi_i$ ) was 0.13 p-equiv/cm<sup>2</sup>. sec. The standard error (S.E.) of a single determination was 0.10 and the standard error of the mean (S.E.M.) was 0.01.

TABLE 1  
COMPARISON OF INFLUXES AND EFFLUXES IN SINGLE CELLS OF *C. AUSTRALIS*

Cell	Influx $\phi_i$ (p-equiv/cm <sup>2</sup> . sec)	Efflux $\phi_o$ (p-equiv/cm <sup>2</sup> . sec)	Temperature (°C)
1	0.13	0.39	26
2	0.14	0.43	26
3	0.22	0.33	25
4	0.09	0.17	21
5	0.08	0.10	21
6	0.08	0.10	21
7	0.15	0.18	21

With 28 cells the mean efflux into A.P.W. ( $\phi_o$ ) was 0.32 p-equiv/cm<sup>2</sup>. sec  $\pm$  0.21 (S.E.) or  $\pm$ 0.04 (S.E.M.). These means are significantly different ( $P < 0.001$ ) but the variability of the fluxes is so large that a significant difference between

TABLE 2  
VACUOLAR CONCENTRATION OF POTASSIUM, SODIUM, CALCIUM, AND CHLORIDE IN *C. AUSTRALIS*  
CELLS FROM FIELD POND WATER OF THE COMPOSITION SHOWN  
Number of estimations given in parenthesis

	Date	Potassium Concn. (mN)	Sodium Concn. (mN)	Chloride Concn. (mN)	Calcium Concn. (mN)
<i>C. australis</i> cells	24.vii.59	86 $\pm$ 3 (8)	49 $\pm$ 1 (8)	—	—
<i>C. australis</i> cells	7. x.59	74 $\pm$ 2 (10)	47 $\pm$ 2 (10)	106 $\pm$ 12 (10)	2.6*
Field pond water		0.06	2.2	2.4	0.16

\* Vacuolar sap of 10 cells pooled.

$\phi_i$  and  $\phi_o$  could be demonstrated only with a large number of measurements. The temperature varied from 20–27°C but was constant during any one determination; the light was sky light, or artificial light of the same approximate intensity.

When  $\phi_i$  and  $\phi_o$  could be compared under similar conditions and in the same cell, efflux was generally in excess of influx so that the cells were not in a steady

state as far as their vacuolar sodium content was concerned. This is shown in Table 1 which lists the results of several experiments on different occasions. In all cases  $\phi_o > \phi_i$ . This is probably connected with the change in external medium from natural pond water to A.P.W. In these experiments  $\phi_i$  is the mean influx over the time used to label the cells enough to make efflux measurements, i.e. 3–14 days, while  $\phi_o$  is the mean efflux in the 12–24-hr period following subsequent rinsing. However,  $\phi_i$  under these conditions is not very different from that measured over 4–6 hr.

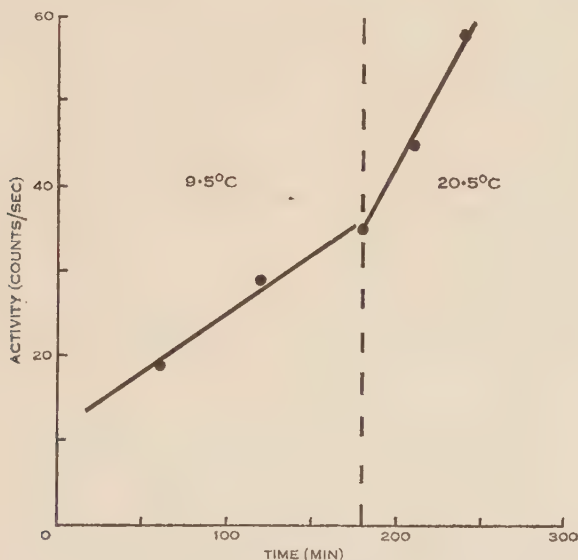


Fig. 1.—Increase in vacuolar radioactive sodium plotted against time for a single cell initially at 9.5°C and later at 20.5°C.

Some analyses of the vacuolar sodium and potassium concentrations of cells of *C. australis* fresh from natural pond water are given in Table 2. The vacuolar concentration of chloride in other analyses was generally somewhat less than the sum of that of potassium and sodium. There was almost no change in  $[K_v]$  and  $[Na_v]$  after soaking cells for up to 7 days in A.P.W.

#### (b) Effect of Temperature on Influx and Efflux

Figure 1 shows the comparative rates of increase in sap activity at 20.5°C and 9.5°C in a single cell in light conditions. The rates corresponded to fluxes of 0.37 and 0.12 p-equiv/cm<sup>2</sup>. sec, respectively. The mean  $Q_{10}$  of  $\phi_i$  in eight experiments was  $2.5 \pm 0.25$  (S.E.M.). It was similar in light and dark. Figure 2 shows the comparative rates of loss of vacuolar radioactive sodium, from a different cell, plotted as counts/min. hr against time for temperatures of 20 and 10°C. These losses of radioactivity correspond to effluxes of 0.38 and 0.18 p-equiv/cm<sup>2</sup>. sec respectively, and therefore the  $Q_{10}$  was 2.1.

(c) *Influx of Sodium as a Function of Sodium Concentration*

The influx of sodium from A.P.W. into the vacuole, in the light and at about 20°C was measured in several external concentrations of sodium ranging from 0.2–5 mN. Collected observations are given in Figure 3, in which are plotted the means and S.E.M. of at least 20 cells at each concentration.  $\phi_i$  was approximately constant up to an external sodium concentration,  $[Na_o]$ , of 2 mN but a large increase was observed in all experiments (of about 4–6 hr duration), when  $[Na_o]$  was 5 mN.

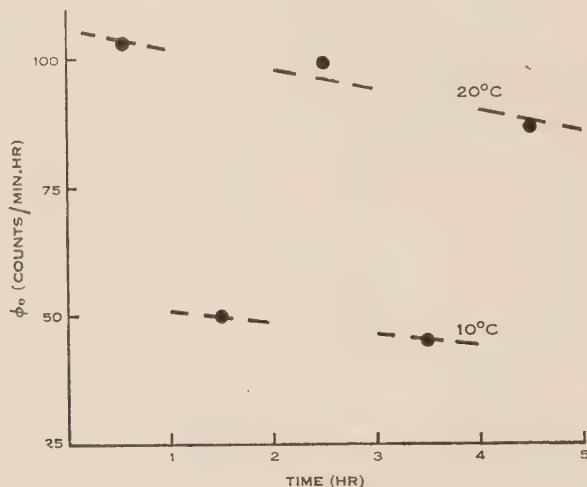


Fig. 2.—Rate of loss of vacuolar radioactive sodium in hourly intervals plotted against time. The temperature alternated between 20°C and 10°C in each hour.

(d) *Fluxes of Sodium in Light and Dark*

The vacuolar influx and efflux of sodium are both larger in the light than in the dark by a factor of greater than 1.5. This is shown in Figures 4 and 5. The possibility that the decrease in  $\phi_i$  in the dark was due to lack of aeration was tested by bubbling the external solution with nitrogen and air, both in light and dark, and the results are indicated in Figure 4. No significant difference was observed between the treatments with nitrogen and air. Table 3 lists some other comparisons between light and dark, in experiments in which saps from different sets of cells were measured at the end of a period of about 4 hr. In other experiments the significance of the difference was occasionally at the level of  $P = 0.1$ , owing to the use of smaller samples.

(e) *Effect of Current Flow on the Fluxes of Sodium*

Single cells were mounted so that "Perspex" stocks divided the length into two equal parts which were insulated from each other by means of "Vaseline" (Fig. 6). Direct current was then passed through the cell by means of Ag|AgCl electrodes dipping into each end of the trough which contained labelled A.P.W. for influx measurements or inactive A.P.W. for efflux measurements.

(i) *Influx*.—The effect of applied current on the rate of increase of vacuolar radioactivity in the light and at 20°C is shown in Figure 7. The two cells had mean influxes of 0.26 and 0.05 p-equiv/cm<sup>2</sup>. sec. The dotted lines indicate the expected influx if the sodium ion carried all the current inwards.\* A small positive effect of current was found but it is likely that the sodium ion carries less than 5 per cent. of the inward current.

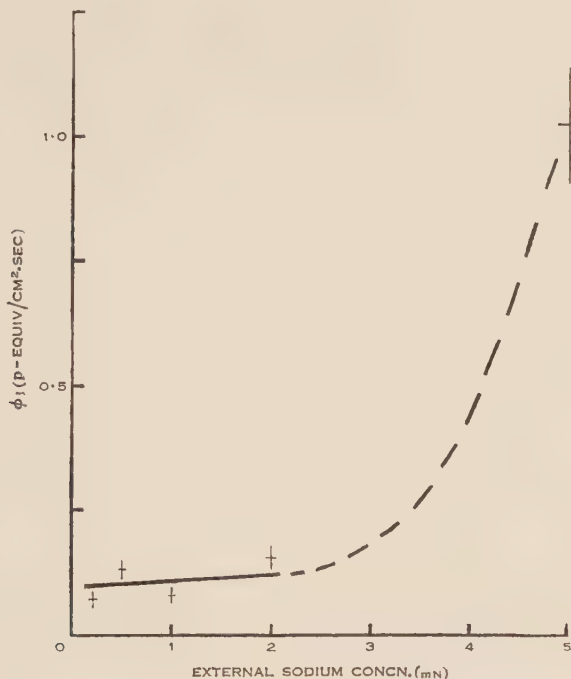


Fig. 3.—Influx of sodium into the vacuole plotted against external sodium concentration. The standard error of the mean of 20 or more cells is given by half the height of the symbols.

An equal loss of activity from the vacuole at the end of the cell in which positive current is being carried outwards would not be expected since the specific activity of the vacuole is very much less than that externally. The assumption that the current passes through the vacuole rather than the cell wall or cytoplasm will be discussed below.

(ii) *Efflux*.—The effect of a current of  $10^{-7}$  A on the loss of activity from a cell which had previously been loaded with Na\* and rinsed for 40 hr with A.P.W. is shown in Figure 8. As in the experiments on effect of current on influx, current was passed in one area of the cell, along the section insulated with "Vaseline", and out at the other end. The areas at each end were usually 0.5–1.0 cm<sup>2</sup> and

\* 96,400 coulombs = 1 equivalent. Therefore  $3 \times 10^{-7}$  A/cm<sup>2</sup> corresponds to a flux of 3.1 p-equiv/cm<sup>2</sup>. sec.



the insulated area about  $0.5 \text{ cm}^2$ . It can be seen that there is no appreciable difference in rate of loss of radioactivity between the segments of the cell kept positive and negative, and the rates are about the same whether current flows or not. The

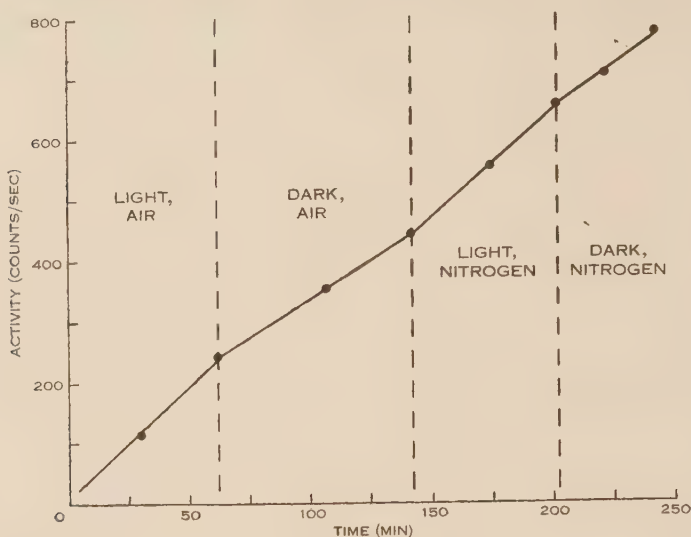


Fig. 4.—Increase in vacuolar sodium activity in a single cell plotted against time for the conditions indicated. The mean influx in the light was  $0.42$  and in the dark  $0.29$  p-equiv/ $\text{cm}^2$ . sec at  $18^\circ\text{C}$ .

high efflux from the end made negative, on the initial passage of current, was found in some experiments and was probably due to residual  $\text{Na}^+$  in the wall and cytoplasm being quickly moved out by the initial current. In subsequent periods of

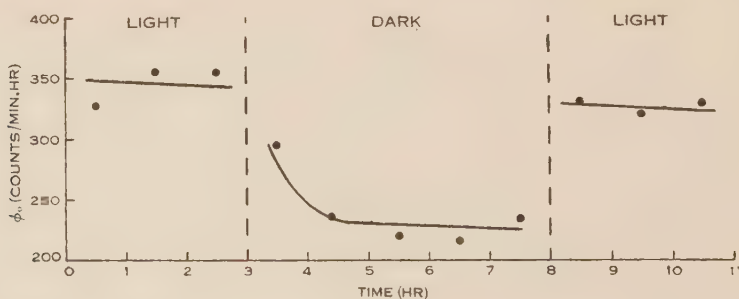


Fig. 5.—Efflux of sodium from the vacuole of a single cell in hourly intervals, in the light and dark. Efflux was  $0.38$  (light) and  $0.26$  (dark) p-equiv/ $\text{cm}^2$ . sec at  $26^\circ\text{C}$ .

current, such effluxes were absent, although the vacuolar  $\text{Na}^+$  content was still high. In Figure 8 the observed  $\phi_o$  was  $0.12$  p-equiv/ $\text{cm}^2$ . sec from both ends of the cell compared with an expected  $1.04$  from the end kept negative, on the assumption that sodium carried all the current.

In these experiments there were three current paths along the cell:

- (1) Along the cell wall (i.e. outside the protoplast).
- (2) Along the cytoplasm (i.e. outside the vacuole).
- (3) Along the vacuole.

It is assumed above that most of the current flows along the vacuole (i.e. through the plasmalemma and tonoplast). A direct measurement was made of the resistance of path (1) in an arrangement similar to that used to measure the effect

TABLE 3

COMPARISON OF MEAN VACUOLAR INFLUXES IN LIGHT AND DARK IN *C. AUSTRALIS* CELLS IN ARTIFICIAL POND WATER

Number of determinations given in parenthesis

Expt. No.	Temp. (°C)	Mean Vacuolar Influxes (p-equiv/cm <sup>2</sup> . sec)		Differences Significant at:
		Light	Dark	
1	26	0.17 ± 0.015 (6)	0.075 ± 0.008 (6)	$P < 0.001$
2	24	0.12 ± 0.026 (6)	0.031 ± 0.012 (8)	$P < 0.01$

of current on flux. An isolated cell wall was fitted tightly on a slightly tapered glass rod, and placed in position on the "Perspex" stocks. A typical resistance measured was 900 k $\Omega$ , while the intact cell in the same position gave 90 k $\Omega$ . It appeared therefore that about 10 per cent. of the applied current flowed along path (1) and had no effect on the tonoplast flux. The relative resistances of paths

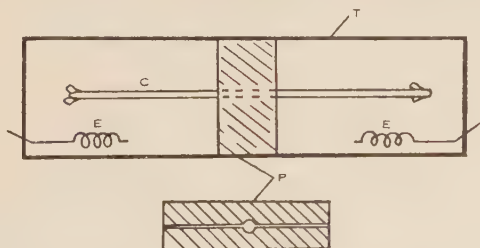


Fig. 6.—Experimental arrangement used when investigating the effect of electric currents on fluxes: *T*, wax trough; *C*, cell; *E*, electrodes; *P*, "Perspex" stocks.

(2) and (3) can be estimated. If the cytoplasm has, as is likely, the same resistivity as the sap (in these cells approx. 50  $\Omega$  cm), its resistance would be some 30 times the resistance of the sap. Unless then the tonoplast resistivity is very high (Walker (1957) found it to be very low in *Nitella*) the resistance of path (2) will be much higher than that of path (3). It can be concluded then that more than 80 per cent. of the applied current crosses the tonoplast.

(f) *Electric Potential of the Vacuole*

In two series of experiments the potential difference between the vacuole and external medium (A.P.W.) was measured in cells which had been living in A.P.W. for about 2 weeks. A glass microelectrode of end diameter about  $5\mu$ , filled with 0.3N KCl, was pushed into the vacuole with the aid of a screw micromanipulator. The potential difference was measured to within  $\pm 0.5$  mV with a valve electrometer of high input resistance. In two series of 10 cells each, which differed in pretreatment, the mean potential difference was as given in Table 4. The sodium and potassium concentrations in the vacuole of the same cells were measured following withdrawal of the microelectrode, during which an insignificant amount of vacuolar sap was lost. The significance of these results is discussed below.

TABLE 4

MEAN ELECTRIC POTENTIAL OF THE VACUOLE AND THE VACUOLAR CONCENTRATIONS OF SODIUM AND POTASSIUM IN *C. AUSTRALIS* CELLS IN ARTIFICIAL POND WATER

Means and standard error of the means of the results from 10 cells in each experiment are given. The last two columns give the vacuolar activity (mN) expected assuming electrochemical equilibrium

Expt. No.	Vacuole Potential (mV)	[Na <sub>v</sub> ] (mN)	[K <sub>v</sub> ] (mN)	[Na <sub>o</sub> ]exp(-EF/RT)	[K <sub>o</sub> ]exp(-EF/RT)
1*	$-157 \pm 1.5$	$57 \pm 3$	$64 \pm 4.5$	490-560	49-56
2†	$-161 \pm 3$	$50 \pm 4$	$68 \pm 2.5$	540-700	54-70

\* Plants soaked for 7 days and single cells therefrom soaked for a further 8 days in artificial pond water.

† Plants soaked for 14 days and single cells therefrom soaked for a further day in artificial pond water.

## IV. DISCUSSION

(a) *Mean Fluxes*

The assumptions on which the measurements of vacuolar fluxes rest need to be examined. All the above estimates are based on two sorts of measurement:

- (i) Rates of increase of radioactivity either in the vacuole (influx) or medium (efflux); and
- (ii) Specific activities of the medium (influx) and vacuole (efflux).

The relations between the fluxes and these quantities have been assumed to be, for unit surface area:

$$\text{Influx} = (1/s_o).dY_v/dt, \dots\dots\dots(1)$$

$$\text{Efflux} = (1/s_v).dY_o/dt, \dots\dots\dots(2)$$

where  $s_{o,v}$  and  $Y_{o,v}$  are specific activities and radioactivities per unit volume in the medium and vacuole respectively. Thus it is assumed that the specific activity

of the cytoplasm,  $s_c$ , rather quickly becomes  $s_o$  (in influx measurements) or zero (in efflux measurements). This involves the assumption that  $\phi_{ic} \gg \phi_{iv}$  and  $\phi_{oc} \gg \phi_{ov}$ . This was found to be so for both potassium and sodium in cells of *Nitellopsis* (MacRobbie and Dainty 1958), but not for potassium in *Nitella axillaris* (Diamond and Solomon 1959) where it was found that in the steady state the specific activity of potassium in the "cytoplasmic non-free space" was only 27 per cent. of  $s_o$ .

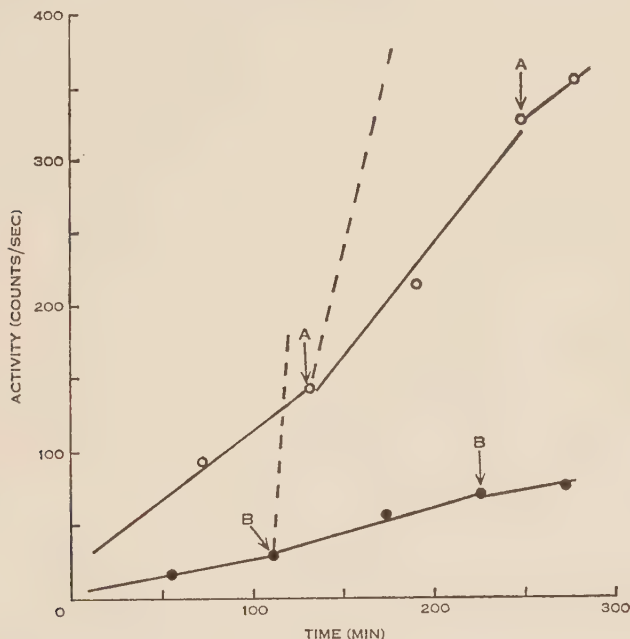


Fig. 7.—Effect of applied current on the vacuolar influx of sodium in two *C. australis* cells. Currents of  $10^{-7}$ A (A) or  $3 \times 10^{-7}$ A (B) were switched on and off where indicated by the arrows. The dotted lines show the expected influxes, assuming all current was carried by the sodium ion.

The way in which our estimates of  $\phi_v$  would differ from those calculated from equations (1) and (2), if in fact  $\phi_c \sim \phi_v$ , can be seen from the following considerations. Referring to Figure 9, for each square centimetre of cell surface:

$$dY_v/dt = \phi_{iv}^* - \phi_{ov}^* = s_c \phi_{iv} - s_v \phi_{ov}, \quad \dots \dots \dots (3)$$

and

$$\begin{aligned} dY_c/dt &= \phi_{ic}^* - \phi_{oc}^* - \phi_{iv}^* + \phi_{ov}^* \\ &= s_o \phi_{ic} - s_o (\phi_{oc} + \phi_{iv}) + s_v \phi_{ov}. \end{aligned}$$

In the steady state  $dY_c/dt = 0$ , whence

$$s_c = (s_o \phi_{ic} + s_v \phi_{ov}) / (\phi_{oc} + \phi_{iv}). \quad \dots \dots \dots (4)$$

Combining (3) with (4), and neglecting  $s_v$  in comparison with  $s_o$

$$\phi_{iv} = \frac{1}{s_o} \cdot \frac{dY_v}{dt} \left( \frac{\phi_{oc} + \phi_{iv}}{\phi_{ic}} \right), \quad \dots \dots \dots (5)$$



that is, the influx calculated according to (1) should be increased by a factor  $(\phi_{oc} + \phi_{iv})/\phi_{ic}$ . In a similar way it can be shown that the efflux is increased by a corresponding factor.

No estimates of cytoplasmic sodium fluxes were made because of the apparently small amount in the N.F.S. in *Chara* cells, but independent estimates of the permeability of the surface membrane from resistance measurements (Hope and Walker, unpublished data) suggest that  $\phi_{ic}$  or  $\phi_{oc} = 1\text{--}2$  p-equiv/cm<sup>2</sup>. sec. If this is so our present fluxes are underestimated by 20–30 per cent. and become about 0.17 (influx) and 0.4 (efflux). This vacuolar influx is of such a magnitude that the half-time for specific activity equilibrium between the vacuole and medium is about 1400 hr.

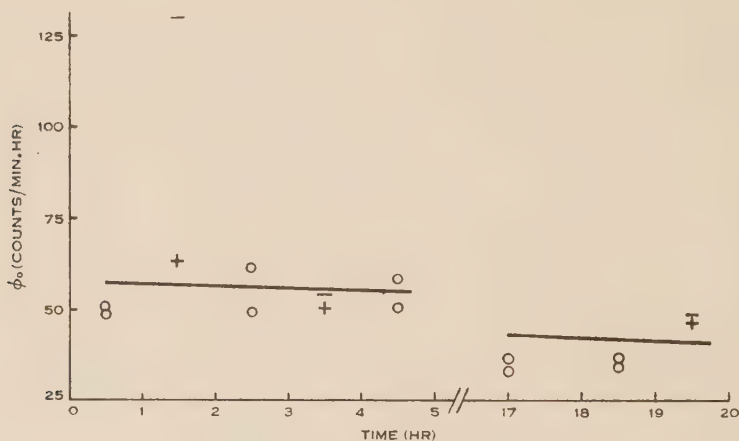


Fig. 8.—Effect of applied current on the vacuolar efflux of sodium from a single cell. Points plotted are activity lost in hourly intervals from the segment of the cell in the pool of solution made positive (+), negative (−), or with no current flowing (O).

#### (b) Effect of Light, Temperature, and Aeration

It was shown that the influx and efflux of sodium across the tonoplast are similarly affected by light and by change of temperature. Thus the influx and efflux seem to be similar processes. The high value of  $Q_{10}$ , 2–2.5, could result from passive diffusion across a high potential barrier (Danielli 1952), or from transport controlled by chemical reactions.

Evidence has been presented here which indicates a connection between sodium transport across the vacuole and a photosynthetic, non-oxidative part of the cell metabolism. This evidence comprises the effect of light in increasing the fluxes, and the absence of an effect on influx of a nitrogen atmosphere in the dark. The mechanism remains to be elucidated.

The variation of sodium influx across the tonoplast with external concentration may reflect a variation of cytoplasmic concentration. This would imply a sudden increase of sodium concentration in the cytoplasm as the external concentration is increased from 2 to 5 mN. Such an increase could result from a sudden

increase in the sodium permeability of the outer membrane, or from saturation of a sodium extrusion pump at the outer membrane.

### (c) Effect of Current on Flux

It has been shown here that the sodium ion contributes little (at most a few per cent.) to an electric current flowing across the tonoplast. Assuming that conduction of the current by protons or electrons is improbable, it would be expected that the major vacuolar ions (potassium, sodium, and chloride) would carry the current. The vacuolar concentrations of these ions are comparable (Table 2) and so indeed are their fluxes across the tonoplast (MacRobbie and Dainty 1958; Diamond and Solomon 1959; and this paper). Each of the three ions might therefore be expected to carry a substantial proportion of the current.

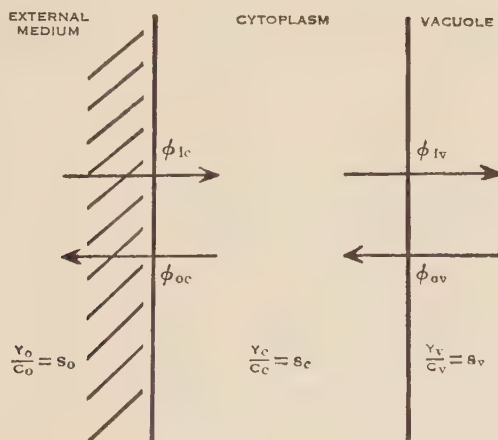


Fig. 9.—Fluxes and specific activities in a three-phase system consisting of external medium, cytoplasm, and vacuole.

The small proportion carried by sodium could result from a sodium transport mechanism in which neutral complexes rather than ions cross the membrane. An alternative explanation, however, is suggested by the discrepancy mentioned in the Introduction. MacRobbie and Dainty (1958) calculated a tonoplast resistance of  $250 \text{ k}\Omega \text{ cm}^2$  for *Nitellopsis obtusa*.

They used their measured ion fluxes, assumed them to be passive (except the chloride influx), and presumably related flux with electric resistance (Hodgkin 1951) by:

$$\begin{aligned} G_j &= (z_j F^2 / RT) \phi_j, \\ G_m &= 1/R_m = \sum G_j, \end{aligned} \quad \dots \dots \dots (6)$$

where

- $R_m$  = membrane resistance ( $\Omega \text{ cm}^2$ ),
- $G_m$  = membrane conductance ( $\text{mho cm}^{-2}$ ),
- $G_j$  = contribution of  $j$ th ion to membrane conductance,
- $\phi_j$  = equilibrium flux of  $j$ th ion across membrane ( $\text{equiv/cm}^2 \cdot \text{sec}$ ),
- $z_j$  = valence of  $j$ th ion.

and  $F$ ,  $R$ , and  $T$  have their usual significance. This relationship holds only for ions in equilibrium across the membrane: it assumes that the ions move independently under the influence of thermal agitation and electric potential gradients.

However, the resistance of the tonoplast of a *Nitella* species is more than an order of magnitude lower (Walker 1957) than the calculated value of MacRobbie and Dainty (1958).

This discrepancy would be reduced, and the present result for sodium explained, if either potassium or chloride contributed to a much higher conductance than one would calculate from their equilibrium fluxes, using equation (6). This is possible if the ion in question crosses the membrane through long narrow pores, as proposed by Hodgkin and Keynes (1955) for potassium in the squid axon membrane. They suggested pores so narrow that ions could not pass each other, but were constrained to move in file. Ion movements are then no longer independent of each other, as required by equation (6). The resistance calculated from the ion flux is then reduced below the above value by a factor  $n$ , where  $n$  is about equal to the number of ions in the file:

$$G_j = (nz_j F^2 / RT) \phi_j. \quad \dots\dots\dots (7)$$

If then the tonoplast of cells of the Characeae contains such pores, specific for potassium or for chloride ions, and containing say 10 ions, the resulting low resistance would help to explain the sodium result presented here and the discrepancy discussed.

#### (d) *Vacuolar Ionic Activities*

At the moment there is no firm basis for distinguishing between potassium and chloride as the ion in pores. However, if the view of MacRobbie and Dainty (1958) that the concentration of chloride in the cytoplasm is much lower than that in the vacuole is accepted, it may be concluded that it is potassium. The ion traversing the pores must be nearly in equilibrium across the tonoplast for the  $n$ -fold relationship between resistance and flux to hold. It has been found in this work that potassium is nearly in electrochemical equilibrium between external medium and vacuole, and may be expected therefore to be nearly in equilibrium across the tonoplast. MacRobbie and Dainty (1958) and Gaffey and Mullins (1958) also found that of the three main vacuolar ions, potassium was nearest to electrochemical equilibrium.

Sodium, calcium, and chloride are definitely not in electrochemical equilibrium in *C. australis* cells (Tables 2 and 4; see also Walker 1957, 1958). The chloride concentration in the vacuole is much greater (100–150 mN) than the equilibrium value (2  $\mu$ N) predicted from the external concentration and the electric potential difference. The concentrations of sodium and calcium in the vacuole are much less than the corresponding equilibrium values. Thus an active sodium-extruding pump suggested for plant cells by Hope and Robertson (1953) and demonstrated in *Nitellopsis* by MacRobbie and Dainty (1958) is probably in operation in *Chara* somewhere between the vacuole and medium. The site of this pump is almost certainly at the outer membrane (as in *Nitellopsis*) because it is here that the electrochemical gradient is directed inwards. This is because the electric potential differ-

ence is found almost entirely at this interface (Walker 1955) and the cytoplasmic concentration is almost certainly less than the equilibrium value calculated from this potential difference, (see Table 4).

#### V. ACKNOWLEDGMENTS

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# THE METABOLISM OF PHLOEM ISOLATED FROM GRAPEVINE

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## Summary

Small slices of phloem tissue have been isolated from grapevine canes and cultured in sugar-buffer solutions, and measurements made of the respiration rate and of the changes of sugar concentration in the tissue. Calculations from these measurements of the permeability of the tissue to sucrose, and of the rate of sucrose synthesis from glucose and fructose, show that the phloem is not very different from other plant tissue in either activity. The bearing of these findings on our knowledge of the translocation of carbohydrates by the phloem is discussed.

## I. INTRODUCTION

The unique structure of phloem has not been shown to be related to its unique capacity for rapid translocation of sugars, nor, in spite of various attempts, have unusual features been found in its physiology which might be related to its capacity for transport. There was a period when plant physiologists believed that sieve tubes were completely permeable to solutes and therefore could not be plasmolysed, but it has been shown recently (Currier, Esau, and Cheadle 1955) that if sufficient care is taken in the preparation of phloem slices, the sieve tubes can be plasmolysed and deplasmolysed repeatedly. Their permeability to solutes cannot therefore be as high as was thought. Very high respiration rates for excised vascular bundles of *Plantago* have been reported by Kursanov and his colleagues (Kursanov 1956) who claimed that this was related to their capacity for transport. Other workers have found difficulty in repeating this work, and no one else has recorded rates of anything like the maximum value given by Kursanov ( $5000 \mu\text{l CO}_2/\text{g fresh wt./hr}$ ). For a review of the early work on this aspect of the problem, see Esau, Currier, and Cheadle (1957). Willenbrink (1957) has measured the respiration of excised vascular bundles of *Pelargonium*, and Ziegler (1958) those of *Heraclium mantegazzianum*, both recording rates of  $\text{CO}_2$  production around  $220 \mu\text{l/g fresh wt./hr}$ .

A principal difficulty in all studies of phloem has been that it is very easily damaged, and most statements about its anatomy and physiology have been criticized on the grounds that cutting the tissue upsets its organization by the release of tensions and pressures. The preparation (by Currier, Esau, and Cheadle 1955) of phloem sections sufficiently undamaged to permit the first observations of plasmolysis seemed to us to offer the possibility of making physiological measurements on phloem that had suffered a minimum of damage. The respiration rate of the isolated tissue is of interest in view both of the discrepancy between the results of Kursanov and those of Willenbrink and Ziegler, and also of the general uncertainty about

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the energy supply to the process of translocation. The permeability of the phloem cells to sugars and particularly to sucrose, the translocated sugar, is of interest in any consideration of movement of sugars through the phloem or between phloem and surrounding tissue. Finally, any inferences that can be drawn about enzymes present in the tissue and their activities will provide facts that may help in constructing hypotheses to explain the translocation process.

Another consequence of this sensitivity of the phloem to damage is that the systems on which we may study the translocation process are always complicated by other processes. Nothing simpler than an intact plant with some defining cut or excision has been found to carry on translocation unimpeded and yield data of rates and quantities and paths of movement. The experimental investigation of the process would be much simplified if some simple system could be found that would translocate sugar, and, with this in mind, attempts were made to measure translocation along the short pieces of phloem prepared like those of Currier and his colleagues.

## II. METHODS

### (a) *Material*

Pieces of phloem were obtained from canes of *Vitis vinifera* (L.) in a way very like that used by Currier, Esau, and Cheadle (1955) in isolating tissue for plasmolysis studies. A 1-ft length from the middle of a current season's cane was placed immediately on cutting in the 0.25M sucrose-buffer (Currier, Esau, and Cheadle 1955). From the middle internodes of this piece, short pieces were cut under buffer solution with a jeweller's saw; and from these short pieces tangential sections were cut, still under buffer solution, at a thickness of 100  $\mu$  on a small horizontal microtome. These sections comprised phloem with a strip of periderm on either side. The strips of periderm were easily detached, leaving a piece of phloem (sieve tubes, parenchyma, and fibres) about 2 by 0.5 cm, and 100  $\mu$  thick, weighing about 14 mg. The bands of sieve tubes were separated by bands of phloem rays (Fig. 1). Up to six sections containing phloem tissue could be cut along one radius of the cane, and of these only the third and fourth were used.

Ten to 30 of these slices were cultured in 10 ml of a solution consisting of the sugar-phosphate buffer to which was added sulphanilamide (0.001M). All buffer solutions had an osmotic pressure equivalent to 0.25M sugar, and when the metabolic sugar concentration was varied, the remaining osmotic pressure was made up with mannitol. Buffers containing mannitol as the only sugar were used in some experiments in which the behaviour of the tissue was studied in the absence of external substrates.

### (b) *Estimation of Sugars*

Sugars in the tissue slices were estimated thus: a sample of slices was withdrawn from the culture, rinsed in mannitol buffer and in distilled water, and placed in hot 70 per cent. ethanol. Two further extractions were made with 70 per cent. ethanol, and the pooled alcohol solutions evaporated and chromatographed on acid-washed Whatman No. 3 paper with the descending eluent ethyl acetate-pyridine-water (80 : 20 : 10 v/v) for about 20 hr. The spots corresponding to suc-

rose, glucose, and fructose were eluted from the chromatogram and determined by the copper-reduction method of Wager (1954). The sucrose was hydrolysed before estimation by holding it at 100°C for 20 min in 0.01N HCl.

### (c) *Respiration*

Two types of respiration measurements were made on the phloem slices. In one set of experiments, the slices were aerated in small glass tubes and the carbon dioxide content of the effluent gas measured in an infra-red gas analyser (IRGA) by comparison with an identical stream which was passing through a similar tube

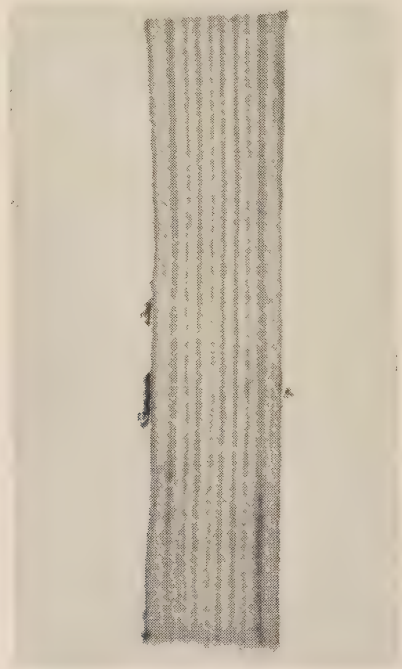


Fig. 1.—Slice of phloem tissue like those used for the experiments. The pale bands consist of sieve tubes and fibres, the dark bands of ray parenchyma. The strips of periderm on either side have been removed.

with no tissue. In the other set of experiments, the carbon dioxide output was measured in a conventional Warburg manometer. Values obtained in the two systems for the same set of slices agreed to within 5 per cent.

## III. RESULTS

### (a) *Respiration*

In 0.25M sucrose, glucose, or fructose buffer, the rate of evolution of carbon dioxide, as measured in either the IRGA or the Warburg manometer, was 220–230  $\mu$ l/g fresh wt./hr. These values are not extraordinarily high for excised plant

tissue, and are consistent with those of Willenbrink (1957) and Ziegler (1958). Both these workers found the carbon dioxide production of the vascular bundles to be much higher than that of the neighbouring parenchyma, but in the vine cane there is, of course, no neighbouring parenchyma for comparison. The xylem, cut in similar sections on the same radius, gives a carbon dioxide output of the same order as that of the phloem. This also agrees with Ziegler's work on *Heracleum*, where the xylem and phloem from a bundle hardly differed in their respiration rates.

(b) *Changes in the Sugar Content of the Phloem after Cutting*

The changes in the sugar contents of the phloem slices in aerated mannitol buffer are shown in Figure 2. There is present on cutting about 2.5 mg/g fresh wt. of sucrose and about 0.5 mg/g fresh wt. of each of fructose and glucose, a point to which we shall return in Section IV. The pattern shown in Figure 2 of falling

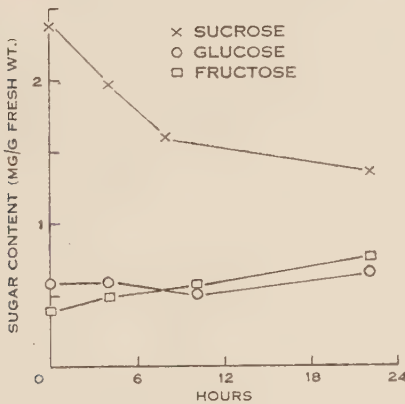


Fig. 2

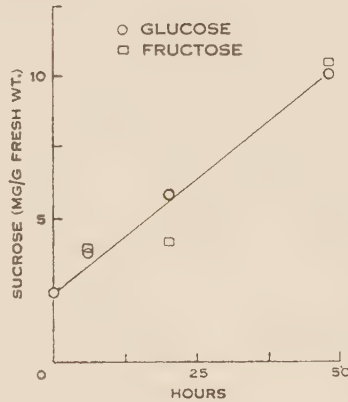


Fig. 3

Fig. 2.—Changes in the sugar content of the phloem during culture in mannitol buffer. The initial rate of sucrose loss corresponds to an output of respiratory carbon dioxide of 82  $\mu$ l/g fresh wt./hr.

Fig. 3.—Synthesis of sucrose: the rise of sucrose content of the phloem in glucose and fructose buffer solutions. The slope of the line corresponds to a rate of synthesis of 0.2 mg/g fresh wt./hr.

sucrose, and slightly rising hexose concentration, is typical of other excised plant tissue in the first hours (James 1953). The initial slope of the sucrose curve in this graph corresponds to a carbon dioxide output of about 82  $\mu$ l/g fresh wt./hr. Direct measurement of this in the experiment quoted above gave about 90 of the same units.

(c) *Synthesis of Sucrose*

If the cultured phloem tissue is supplied with either glucose or fructose in the buffer solution, sucrose is rapidly synthesized in the tissue. The sucrose content rises from the initial value of about 2.5 mg/g fresh wt. as shown in Figure 3. The rate of increase is about 0.2 mg/g fresh wt./hr.



*(d) Permeability of the Phloem to Sucrose*

From the measurements of increase in sugar content of the tissue cultured in sucrose buffer, we can estimate the permeability of the phloem to sucrose. Figure 4 shows the changes in the three sugars with time in four different concentrations

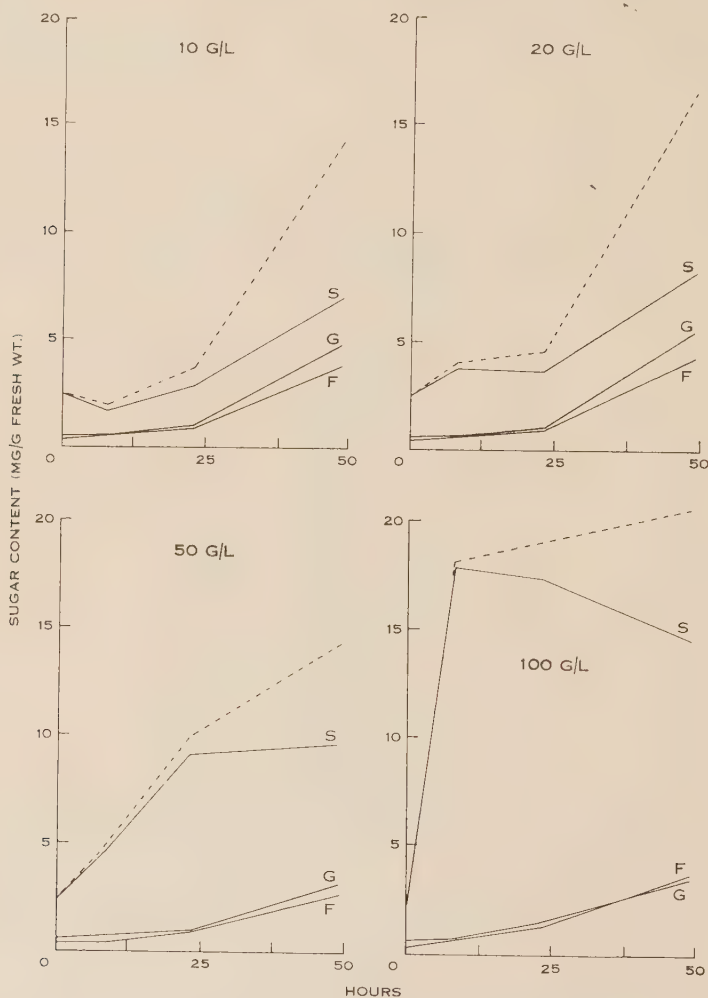


Fig. 4.—Permeability of phloem tissue to sucrose. The changes are shown of the sucrose, glucose, and fructose contents of the phloem when cultured in four different concentrations of sucrose buffer: 10, 20, 50, and 100 g/l. The extra glucose and fructose produced with time are presumably products of sucrose breakdown, and the dotted curves represent the measured sucrose content plus the additional sucrose which would produce the measured increases in glucose and fructose.

of sucrose buffer. The increases in glucose and fructose are presumably due to breakdown of sucrose in the tissue, and the dotted curves are the sucrose values obtained by adding at each estimation the extra glucose and fructose. The initial

slopes give the rates of increase: that in 10 per cent. sucrose buffer represents a permeability of 2 mg/g fresh wt./hr.

(e) *Translocation along Pieces of Phloem*

Attempts were made to reveal transport of sucrose along isolated slices of phloem by feeding  $^{14}\text{C}$ -sucrose to one end of a slice while the other end was supported over a metal slit under which was a Geiger tube. If rates of movement anything like those found for sucrose in intact phloem could be realized in the isolated system, radioactivity should be readily detected at the far end of the slice in a few minutes. We have to record our failure to observe any transport of radioactivity in the slices, just as Ziegler failed to observe any transport along isolated vascular bundles of *Heracleum*.

#### IV. DISCUSSION

The tissue isolated by the technique described and used for the experiments has been referred to as "phloem", and in the morphological sense it is so, but from the physiological point of view it is a heterogeneous tissue not all of which may be concerned with sugar transport. As will be seen in Figure 1, the paler bands of elongated cells are separated by darker bands of isodiametric ray cells which are mostly filled with starch. The paler bands contain many sieve tubes, but also many fibres. The observed reactions may therefore be reasonably described as characteristic of phloem in its broad sense, but not of sieve tubes only since these comprise probably less than half of the bulk of each slice. The observations may therefore be interpreted as related to translocation only in so far as the whole phloem is concerned in translocation, or if the reactions of the sieve tubes alone are important, we must realize that these have been diluted by other cells.

The results presented above are unremarkable. The physiology of slices of phloem is little different from that of other isolated plant tissue in that each of the values obtained can be paralleled by work on other plant parts. A respiration rate of  $220\ \mu\text{l CO}_2/\text{g fresh wt./hr}$  is perhaps a little higher than would be expected of most tissues in sugar buffers, but small compared with values like those recorded for some flower parts ( $800\ \mu\text{l/g fresh wt./hr}$ ). A rate of sucrose synthesis of  $0.2\ \text{mg/g fresh wt./hr}$  is comparable with those recorded by Nelson and Auchincloss (1933) for potato tuber slices,  $0.38$  of these units; by McCready and Hassid (1941) for barley shoots,  $0.35$ ; and by Porter and May (1955) for *Nicotiana*,  $0.2$ . Permeability to sucrose at the rate of  $2\ \text{mg/g fresh wt./hr}$  is smaller than that measured by Weatherley (1953, 1954, 1955) for leaf disks of *Atropa* ( $5$  of these units), but he states that this leaf has an abnormally high permeability. The more usual rate of penetration appears from his data to be around  $1\ \text{mg/g fresh wt./hr}$ . Porter and May (1955) record their permeability data in terms of  $\text{mg}/12\ \text{leaf disks}/8\ \text{hr}$  for *Nicotiana* leaves, but rough estimates of the area-density of tobacco leaves applied to their results yield a value of about  $0.25\ \text{mg/g fresh wt./hr}$  at 5 per cent. sugar in the solution. The phloem may thus be rated as highly permeable to sucrose.

Although nothing remarkable has been revealed about the physiology of phloem tissue, the results are nevertheless of some interest in the wider field of

translocation studies. The mere measurement of the sucrose content of phloem is interesting since all speculations about sucrose movement involve guesses at the concentration of the solution which is moving. Most workers have favoured a concentration of about 20 per cent. sucrose for the translocation solution, arguing from the measured rates of transfer of dry weight, and these estimates have received some support from the measured concentration of sucrose in phloem exudates which are commonly around 17–20 per cent. The question of whether the exudate represents either the contents of the sieve tubes or the translocated solution is still a vexed one, and has not been made any clearer by the fact that whenever measurements of sucrose content have been made on isolated phloem the sucrose is about 1 per cent. of the fresh weight. The present estimate of 2.5 mg/g fresh wt. in freshly isolated phloem is even lower. If sieve tube sap contains 25 per cent. sucrose this must occupy only one-hundredth of the phloem, and if this is so, it is difficult to see where the reserves of sap come from to supply large drops of exudate. From microscopic measurements, the proportion of the phloem occupied by sieve tube lumina has been generally estimated as about one-fifth. There is a paradox here that lies at the basis of the translocation problem.

The measurements of respiration rates of phloem presented in this paper constitute additional evidence that the very high rates reported by the Russians must be regarded with suspicion. Not only is the respiration rate of vine phloem remarkably similar to those of *Pelargonium* and *Heracleum* when supplied with sugar substrate, but when respiring only its endogenous substrates attains the modest figure of 90  $\mu\text{l CO}_2/\text{g}$  fresh wt./hr. It is likely that this is closer to the rate in the intact vine cane, and is unlikely to represent a large part of the energy supply to translocation.

The high permeability of the phloem to sucrose is of some interest in relation to the question of plasmolysis of sieve tubes, for, as Weatherley (1955) points out, a sucrose permeability of this order will seriously alter the values obtained for suction pressure of the tissue by any plasmolytic method using sucrose as plasmolyticum. The values obtained by Currier, Esau, and Cheadle (1955) for suction pressures of sieve tubes may well be too high. They record that grapevine phloem often requires a 2M sugar solution to plasmolyse it, corresponding to a suction pressure of about 150 atm. The results presented above suggest a partial return to the older standpoint of permeable sieve tubes, and may explain some of the early difficulties in plasmolysing them.

Our failure to reveal any transport of labelled sucrose along phloem slices and the similar failure of Ziegler seem to indicate that although phloem isolated carefully from the plant can carry on many of its activities (including respiration and sucrose synthesis), and can be plasmolysed, its essential capacity for transport has been destroyed. It is becoming doubtful whether such measurements as those of Kursanov, Willenbrink, Ziegler, and those presented in this paper can ever tell us about those peculiar aspects of phloem physiology that are related to translocation. We have studied excised phloem and found it to be not very unlike other plant tissues. The remaining problem is more difficult, to study the physiology of the phloem while it is still on the plant and actively engaged in translocation.

## V. ACKNOWLEDGMENT

We wish to record our thanks to Dr K. S. Rowan and Mr. G. Everson, Botany School, University of Melbourne, for their help with determinations of respiration rate by means of the Warburg manometer and the infra-red gas analyser; and to the Directors of I.C.I.A.N.Z. for permission to publish this paper.

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# AN INVESTIGATION OF THE POLYSACCHARIDES PRESENT IN SUGAR MILL SYRUPS

By G. K. SUTHERLAND\*

[*Manuscript received March 15, 1960*]

## *Summary*

Two major polysaccharides have been found in syrups obtained from various sources. They were separated by fractional precipitation with ethanol, and the homogeneity of the fractions was checked by moving-boundary electrophoresis. One of the polysaccharides was found in all samples examined and appeared to be a hemicellulose type. The other polysaccharide was a polyglucose of the dextran type, and its appearance was associated with large increases in the viscosity of the syrup. The possible origins of the polysaccharides are discussed.

## I. INTRODUCTION

The isolation and identification of a dextran polysaccharide found in sugar-cane was the subject of a previous report from these Laboratories (Nicholson and Lilienthal 1959). Data gathered at that time showed that there was a variation in the concentration of this polysaccharide during the cane-crushing season. A check on the viscosity of sugar syrups prepared from the cane by crushing, clarification of the juice, and subsequent concentration showed that large increases in the viscosity of syrups occurred at certain times during the sugar-milling season. Since water-soluble polysaccharides present in the sugar-cane should carry through into the syrup, certain syrups were inspected for the presence of polysaccharides.

Moving-boundary electrophoresis has provided in recent years a technique for establishing the degree of homogeneity of a polysaccharide preparation. Fractionation procedures can be evaluated by electrophoretic examination of the fractions, and a suitable electrophoretically homogeneous fraction chosen for characterization. Northcote (1954) has established that borate ion is necessary to give a charge to neutral polysaccharides, while Bernier (1958) has examined several polysaccharides from different soils with this method. Lewis and Smith (1957) have also shown that electrophoresis on glass-fibre paper in strong alkali could indicate a heterogeneity that was not suspected from previous results.

The isolation of individual polysaccharide types from mixtures has been carried out successfully by fractional precipitation with ethanol at a convenient pH (Whistler and Lauterbach 1958). The shape of the fractionation curve has also been used to indicate the homogeneity of the preparations (Whistler and Be Miller 1956; Whistler and Kirby 1956). Optical rotation and identification of the component sugars in the fractions also give some indication of the efficiency of the fractionation procedure (Adams 1957).

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## II. EXPERIMENTAL

(a) *Isolation of Crude Polysaccharide Preparations from Syrups*

Syrups were obtained from various sugar mills in Australia and Fiji throughout the crushing season and stored in the cold until required.

The syrup was diluted with water until its concentration was 60 per cent. (w/v) and 4 volumes of ethanol were added. The precipitate was centrifuged off, the water-soluble fraction dissolved in water, and the solution dialysed for 48 hr in the cold against five changes of water. The dialysis residue was then passed over a cellulosic anion exchanger (see below) to remove protein and most of the colour.

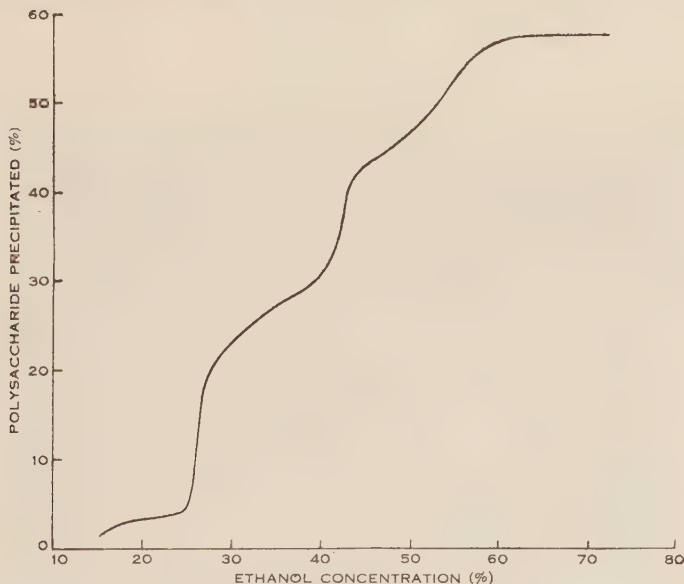


Fig. 1.—Fractional precipitation of a crude polysaccharide preparation from a 2 per cent. aqueous solution at pH 4.5.

The column was washed with water, and the effluent and washings were concentrated at 35–40°C under vacuum until the concentration of dissolved material was approximately 3 per cent. The crude polysaccharide material was then obtained by adjusting the pH to 4.5, precipitating with 4 volumes of ethanol, and drying the precipitate *in vacuo*.

(b) *Cellulosic Anion Exchanger*

This was prepared from ethyl cellulose (Hercules Powder Co., Delaware, U.S.A.) by a method based on that of Peterson and Sober (1956) and adapted for this Department by B. Cortis-Jones (personal communication).

(c) *Fractionation of the Crude Polysaccharide Preparations*

The dried material was broken up and dissolved in water to 1–2 per cent. concentration, and any insoluble material removed by centrifuging. The pH was adjusted to 4.5 with acetic acid, and ethanol added slowly with continual stirring.

Fractions were collected by centrifuging when sufficient material had precipitated, and were dried at 35–40°C under vacuum for several hours.

(d) *Moving-boundary Electrophoresis*

This was carried out with a Perkin-Elmer Model 38A apparatus according to the method of Northcote (1954).

(e) *Paper Electrophoresis*

This was carried out on glass-fibre paper according to Briggs, Garner, and Smith (1956) and carbohydrate zones detected with a solution of *p*-anisidine sulphate in ethanol used as a dip (1 g *p*-anisidine + 2 ml concd. sulphuric acid in 100 ml ethanol—see Fuller and Northcote 1956).

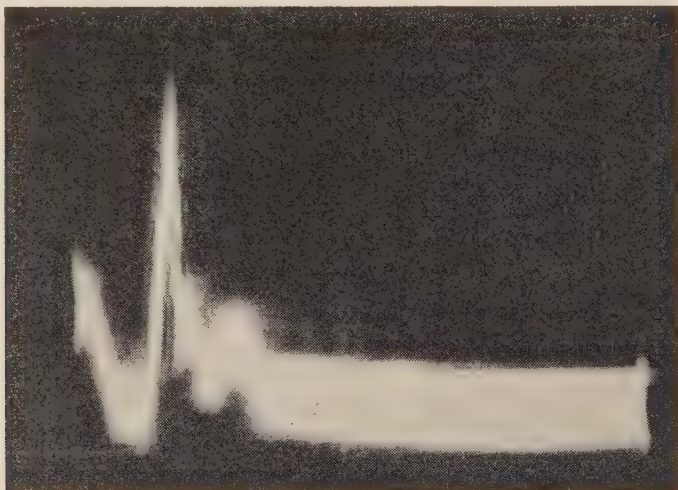


Fig. 2.—Electrophoretic pattern of a crude polysaccharide preparation from sugar syrup: borate buffer (pH 9.3, 10 mA, 1 per cent. polysaccharide); ascending boundary exposed at 50 min.

(f) *Polysaccharide Hydrolysis and Paper Chromatography of Sugars*

Samples (20 mg) were heated at 100°C for 8 hr in 2N sulphuric acid under reflux. The solutions were neutralized with saturated barium hydroxide, centrifuged, and the supernatant dried at 35–40°C under vacuum. The residue was dissolved in 2 ml water, and the appropriate amount (10–50  $\mu$ l) applied to the paper chromatogram with an “Agla” micrometer syringe. Mild hydrolysis to liberate arabinose was carried out in 0.1N oxalic acid at 100°C for  $\frac{1}{2}$ –1 hr and the sugars recovered similarly.

The chromatograms were run in a benzene–butanol–pyridine–water solvent (1 : 5 : 3 : 3 v/v) overnight by descending chromatography (Hathway and Seakins 1958). Other solvents used were ethyl acetate–pyridine–water (8 : 2 : 1 and 2 : 1 : 2 v/v—Whistler and Kirby 1956). The sugars were most conveniently located by

detection with alkaline silver nitrate, adapting the method of Trevelyan, Procter, and Harrison (1950). The chromatogram was dipped in the silver nitrate solution, but the alkaline spray was replaced by dipping in a solution prepared by diluting 50 ml of 40 per cent. sodium hydroxide to 1 l. with ethanol. The chromatogram was then air dried, washed in a fixing solution (10 per cent. sodium thiosulphate + 1.5 per cent. sodium metabisulphite + 15 ml acetic acid per litre of solution) until the background colour disappeared, and finally washed for 1 hr in running water. After this treatment the chromatograms could be preserved for a considerable time. The sugars could also be detected by spraying with *p*-anisidine hydrochloride and heating (Hough, Jones, and Wadman 1950).

TABLE 1  
FRACTIONAL PRECIPITATION OF A 1 PER CENT. AQUEOUS POLYSACCHARIDE MIXTURE

Fraction	Electrophoretic Mobility* (cm <sup>2</sup> sec <sup>-1</sup> volt <sup>-1</sup> )	Ethanol Concn. (%)	Optical Rotation [α] <sub>D</sub> <sup>20</sup>	Sugars Detected†
1	$1.4 \times 10^{-5}$	41.6	+198°	Galactose (m), glucose (M), arabinose (m), xylose (t), rhamnose (t)
2		46.1	+176°	Galactose (m), glucose (M), arabinose (m), xylose (m), rhamnose (t)
3		48.1	—‡	
4		50.0	—‡	
5		53.3	+140°	
6		54.8	+ 93°	
7	$2.5 \times 10^{-5}$	57.6	+ 57°	Galactose, glucose, arabinose, xylose, rhamnose (t)
8		60.0	—‡	

\* pH 9.3 borate buffer.

† M = major, m = minor, t = trace.

‡ Insufficient material.

#### (g) Optical Rotation

This was measured for a 1 per cent. aqueous solution on an ETL-NPL automatic polarimeter, type 143A, using a mercury lamp. This instrument enabled measurements to be made on small amounts of material.

#### (h) Viscosity

Measurements were made on syrups brought to a standard concentration (73 per cent. solids by weight) at 60°C in a Höppler rheoviscometer. The syrup viscosity was compared with the viscosity of a sucrose solution under the same conditions, and used as a preliminary guide in the selection of syrups with various polysaccharide contents.



*(i) Dextrans*

Dextran was prepared from *Leuconostoc mesenteroides*, and a sample of cane dextran was prepared from stale sugar-cane (Nicholson and Lilienthal 1959). The dry solids were obtained by ethanol precipitation and vacuum drying at 40°C.

## III. RESULTS AND DISCUSSION

Precipitation with ethanol from a diluted syrup gave a material with a high ash content. This was reduced to a suitably low figure (1–5 per cent.) by subsequent solution and dialysis. After preparations from several syrups had been investigated, a pattern began to emerge connecting the viscosity of the original

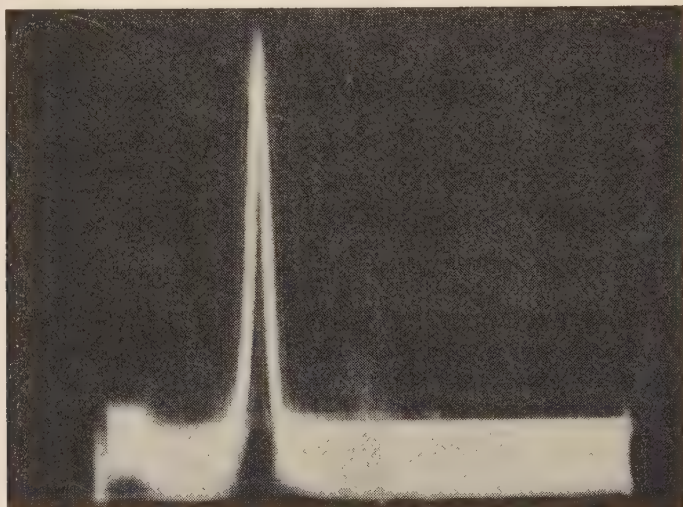


Fig. 3.—Electrophoretic pattern of a polyglucose isolated from the polysaccharide mixture in Figure 2: borate buffer (pH 9.3, 10 mA, 0.75 per cent. polysaccharide); ascending boundary exposed at 120 min.

syrup, the number of components in the crude polysaccharide that were resolved by moving-boundary electrophoresis, and the relative amounts of sugars that appeared on chromatograms of the hydrolysates.

The material from low-viscosity syrups gave chromatographic evidence for xylose, arabinose, glucose, and galactose in rather similar proportions, as well as a trace of rhamnose. The electrophoretic pattern indicated the presence of one main component. The appearance of another component with a slower but sharper boundary was associated with syrups of a slightly higher viscosity.

When the crude polysaccharide material from high-viscosity syrups was examined, a very strong glucose spot appeared on chromatograms of the hydrolysate, as well as the other sugar spots obtained before. The increase in glucose content coincided with the appearance on the electrophoretic patterns of a very

strong peak, corresponding in mobility to the peak which had begun to appear in the preparations from medium-range viscosity syrups. The amount of this component (as judged by electrophoresis) and the amount of glucose found on hydrolysate chromatograms increased with the viscosity of the original syrup.

These results indicated that there were two polysaccharide components that could occur in the syrups. One of these appeared to be related to increases in viscosity noticed during the season. The absence of any blue colour when crude polysaccharide preparations were tested with a dilute iodine solution suggested the absence of starch; nor was there any positive test for uronic acids (Dische 1947).

TABLE 2  
PROPERTIES OF ISOLATED POLYSACCHARIDES AND DEXTRANS

Compound	Optical Rotation* [ $\alpha$ ] <sub>Hg</sub> <sup>20</sup>	Electrophoretic Mobility† (cm <sup>2</sup> sec <sup>-1</sup> volt <sup>-1</sup> )	Sugars Detected‡
Syrup dextran	+198° to +240°	1.2–1.5 × 10 <sup>-5</sup>	Glucose (M), arabinose (t)
Stale cane dextran	+228°	2.6 × 10 <sup>-5</sup>	Glucose
Dextran from <i>L. mesenteroides</i> §	+251°	1.7 × 10 <sup>-5</sup>	Glucose
Syrup hemicellulose	+57° to +98°	2.4–2.5 × 10 <sup>-5</sup>	Galactose, glucose, arabinose, xylose (m), rhamnose (t)

\* 1 per cent. aqueous solution.

† Measured in 0.05M sodium borate solution (Northcote 1954),  $I = 10$  mA, using ascending boundary.

‡ M = major, m = minor, t = trace.

§ This material showed a minor fast electrophoretic component.

The crude material was soluble in 1N sodium hydroxide, and gave very little precipitate with Fehling's solution (Chanda *et al.* 1950), or with cetyl trimethylammonium bromide and sodium borate (Barker, Stacey, and Zweifel 1957). After mild hydrolysis with 0.1N oxalic acid, arabinose only could be detected in chromatograms.

The crude polysaccharide material was fractionally precipitated with ethanol at pH 4.5. This has been suggested as an analytical and a preparative technique by Whistler and Lauterbach (1958). Figure 1 shows the shape of a typical fractionation curve from a crude material. Figure 2 is the electrophoretic pattern of this same polysaccharide mixture. Fractionation at pH 4–5 assisted in the removal of most of the colour. Table 1 shows some characteristics of fractions collected from a typical preparation.

The shape of the fractionation curve indicated that the two main polysaccharides could be separated by this precipitation method. Examination of the

appropriate fractions showed that both polysaccharides could be obtained electrophoretically homogeneous. The fraction which was collected at lower ethanol concentrations (Fig. 3) was essentially a polyglucose, although several precipitations with ethanol did not remove final traces of arabinose from chromatograms of the hydrolysate. The high positive rotation suggests it is a dextran type, although it has a different mobility from the two other dextrans inspected (Table 2). The other polysaccharide is a heteroglycan of the hemicellulose class. Some properties of the two polysaccharides, together with the two dextrans inspected, are included in Table 2. The separated polysaccharides also showed different rates of movement when subjected to electrophoresis using glass-fibre paper.

The presence of two main polysaccharide constituents of sugar syrups has thus been established, one a dextran and the other a hemicellulose. The hemicellulose has been present in all syrups examined and is probably a cell-wall constituent. Nicholson and Lilienthal (1959) showed that an abnormal dextran could form in sugar-cane during a period of delay before the cane is crushed. The dextran which has been found in sugar syrups could well be caused by abnormal metabolism or infection of the cane, if any delay occurs between the burning and the crushing of the cane. This dextran contributes to the abnormally high viscosities observed in some syrups.

#### IV. ACKNOWLEDGMENTS

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# THEORETICAL CONSEQUENCES OF TRUNCATION SELECTION BASED ON THE INDIVIDUAL PHENOTYPE

By B. GRIFFING\*

[*Manuscript received March 7, 1960*]

## *Summary*

Theoretical consequences of truncation selection based on the individual phenotype are examined for the following cases of increasing genetic complexity: (i) an arbitrary number of alleles at a single locus, (ii) an arbitrary number of alleles at each of two linked loci, and (iii) a completely general genetic situation.

The analyses are facilitated by generalizing the concept of hereditary units to include not only the gene but also units of higher levels of organization.

Analyses fundamentally based on higher-order units but with a gene interpretation permit a detailed examination of the consequences of selection and relaxation following selection for the two-locus case. It is shown that the immediate response to selection may be different from that predicted on the basis of gene analysis if an additive  $\times$  additive type of epistasis occurs. However, due to the "mutability" of these higher-order inheritance units, the population mean, on relaxation of selection, decays to that predicted by the gene-analysis approach.

## I. INTRODUCTION

Apart from the abbreviated presentation in Section 7 of the remarkable paper by Kimura (1958), there seems to be no generalized treatment of the theoretical response of a population to continuous artificial selection. However, various intuitive suggestions have been made without an extensive rigorous mathematical treatment. For example, Lush (1948) makes the following comprehensive statement:

"Epistatic variations are in small part transmitted; i.e. they are correlated as between parent and offspring although not as highly correlated as the genic differences are. Therefore we do expect to recover temporarily in the offspring some fraction of whatever epistatically caused phenotypic differences in the parents led to our selecting those parents in the first place. When mating is random, this fraction would be about half of the two-gene epistatic interactions, one-fourth of the three-gene interactions, one-eighth of the four-gene interactions, etc. Therefore only a little of the epistatic variance would be removed unless nearly all epistasis is from simple two-gene and three-gene interactions. The phenotypic gains from selecting for epistatic differences come from distorting the gametic array and soon disappear after selection is relaxed, as the gametic array returns to random. By contrast, the gains from changes in gene frequency are permanent unless and until counter-selection restores the original gene frequency."

Kempthorne (1957, p. 361), when discussing Fisher's "Fundamental Theorem of Natural Selection", also makes an interesting statement which is more explicit with regard to the contribution of specific epistatic variance components. The statement is:

"It is interesting to speculate on the possible extension of the result to the case of more than one locus. It is likely that, if the appropriate  $\lambda$  quantities remain constant, the rate of increase in fitness will involve both additive and additive  $\times$  additive components of genotypic variance of fitness."

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Starting with these intuitive notions, it is now desirable to provide a more rigorous and detailed development of the theoretical responses of a population to artificial selection.

More exactly, the objectives of this study are to discuss some aspects of the general problem of description and prediction associated with truncation selection based on the individual phenotype. The argument will be developed for increasingly complex genetic situations until the generalization is reached which includes any number of alleles at each of an arbitrary number of loci associated in an arbitrary system of linkages. The genes may exhibit any set of dominance and epistatic values.

The generalization of results is made easier by a generalization of the notion of hereditary units to include not only the gene but also units of higher levels of organization. Because of the emphasis which is placed on the notion of generalizing the unit of inheritance, the following points will be discussed in the next three sections:

- (i) The problem posed by the modern concept of the gene and the possibility of considering units of higher levels of genetic organization.
- (ii) The characterization of a random-mating population in equilibrium by different units of inheritance.
- (iii) The generalization of definitions which have been developed for the gene analysis in order that they may be applied to higher-order units.

After these introductory sections, the consequences of truncation selection will be examined when selection is applied to the following genetic populations:

- (i) Genotypes which are generated at a single gene locus—the argument is based on the usual gene analysis.
- (ii) Genotypes which are generated at two gene loci which are linked—the argument is based on a gametic analysis with a gene interpretation.
- (iii) Genotypes which are generated by a completely arbitrary gene system—the argument is based primarily on an individual analysis.

The importance of this subject is considerable for at least two reasons. Firstly, mass selection (i.e. selection based on the individual phenotype) is undoubtedly the most extensively used method of selection in plant and animal breeding. It is, therefore, important to know what are the theoretical consequences of such a selection programme, and to know how predictions of genetic gains may be made.

Secondly, a recent trend in quantitative genetics is to make inferences regarding the phenomenon of "genetic homeostasis" (Lerner 1954), from controlled selection experiments, which are usually conducted with a convenient experimental organism such as *Drosophila*. In these experiments it is often observed that the mean of a population, which has been subject to unidirectional artificial selection, regresses, on relaxation of selection, toward its original position. This phenomenon has been termed genetic homeostasis by Lerner (1954), and it is assumed that this regression is due to the effects of natural selection which act antagonistically to artificial selection.

It is clear that to properly evaluate the effects of natural selection from selection experiments alone (i.e. without fitness tests), it is essential to understand the theoretical consequences of artificial selection and relaxation *in the absence of natural selection*. For example, it will be shown that the contributions of non-zero additive  $\times$  additive  $\times$  etc. components of variance to the responses of selection and relaxation following selection mimic those due to natural selection. Thus, unless the effects from at least one source can be measured, the effects of both are hopelessly confounded and inferences about the contribution of natural selection cannot be made.

#### (a) *Units of Inheritance*

It appears that the modern concept of the gene, which is evolving from intensive studies of complex loci in the entire range of organisms from bacteriophage to man, differs radically from the classical notion of the gene as a single indivisible unit capable of being described unambiguously in terms of recombination, mutation, and physiological activity. It now appears that the chromosomes may be divided, first, into fundamental loci (i.e. limited regions) each of which specifically controls a biological activity. It is probable that each locus contains many mutational and recombinational sites, and, in many instances, linear linkage maps may be obtained for the locus from intralocus recombination data. In some cases, in which pairs of mutants within the same locus can be tested for complementarity, maps of functional regions (cistrons) may be obtained. However, anomalies may arise with the complementation criterion. For example, when mutants have pleiotropic effects, complementation tests may lead to cistrons which are physically discontinuous (Carlson 1959). A particularly lucid summary of the modern concept of the complexities which may exist at a locus is given by Fincham (1959):

"The picture which seems to be emerging, at least from the *Neurospora* work, is of chromosomes segmented into small and apparently quite sharply distinct regions, each concerned, though almost certainly indirectly, in the formation of a particular kind of protein molecule. Within these regions, which I have been calling loci, mutations can occur at many different sites. Different mutant derivatives of the same locus, that is to say alleles, can often interact at meiosis, either by crossing-over in the orthodox sense or by "conversions", to give a wild-type allele, and their cytoplasmic products can sometimes interact to give a wild or semi-wild phenotype in a heterocaryon. Loci of this kind conform to none of the usual definitions of the gene. Yet the most striking and incontrovertible fact about the chromosome as a genetic structure has surely always been its segmentation into regions of highly specific and differentiated function. It is this fact, rather than any segmentation of the chromosome with respect to crossing-over, which has given solidity to the idea of the gene, and which still does so. While the recon becomes vanishingly small, and the cistron tends to fall apart, the gene locus, regarded as a physiologically differentiated segment, still retains a semblance of reality."

The question is how does this modern concept of the gene affect the treatment of quantitative inheritance? More explicitly, the issue is how to characterize the genetic complexity at a locus in order to facilitate the analysis of quantitative inheritance and theoretical problems in plant and animal breeding.

There are at least two methods of representing the genetic situation at a complex locus. To illustrate, consider a locus which has a simplified structure

consisting of only two genetic conditions (mutant and normal) at each of two mutational sites. In the first method, the locus can be subdivided into two subloci, one for each of the mutational sites. This approach yields two sets of alleles, each set being the genetic alternatives at each sublocus. In this case, the gene model for quantitative inheritance must be extended to accommodate position effect which may occur between alleles at different subloci. This, so far, has never been done.

The alternative method is to consider the overall locus as the basic entity, and to regard all possible genetic structures at this locus as the set of multiple alleles. Thus, in the simplified example, the four possible gene states are  $(++)$ ,  $(m_1+)$ ,  $(+m_2)$ , and  $(m_1m_2)$ . These, then, would be regarded as the alleles of the locus. Such a representation avoids the introduction of intralocus position effect because complexities such as the *cis-trans* relations would be absorbed in the dominance parameters. However, a resultant complication of this approach is that mutation of alleles as defined above includes both point mutation in its conventional sense and intralocus recombination. For example, recombination between mutational sites arranged in a *trans* configuration,  $m_1+ / +m_2$ , results in non-parental locus types,  $(++)$  and  $(m_1m_2)$ . It is of course clear that the frequency of such intralocus recombination is low compared with the frequency of recombination between genes at different loci. Hence, it would appear that with the alleles defined as above, the contribution of locus mutation (point mutation and intralocus recombination) would be negligible in most theoretical plant and animal breeding studies.

Assuming that this representation of a locus is satisfactory, it is possible and desirable to extend the notion of hereditary units to higher levels of genetic organization; that is, to the chromosome, gamete, and individual. It will be shown that such an extension is valuable for certain aspects of selection theory.

In a given population there exists a finite number of different configurations for a given set of homologous chromosomes. Each configuration can be considered as an "allele": a macrogene with multiple effects. These alleles are unstable since mutation now includes point mutation, all forms of chromosomal aberrations, and recombination within and between loci along the entire length of the chromosome. To further extend the analogy with the gene concept, each set of homologous chromosomes may be regarded as a multiple allele series at one locus; there being  $n$  loci in all, where  $n$  is the haploid chromosome number. The average effect for a given chromosome can be defined as well as the interactions between homologous chromosomes and the interactions between non-homologous pairs of chromosomes.

In a similar manner, gametes represent a higher level of genetic organization. In this case individual gametes are analogous to alleles and the entire array of gametes in a population is analogous to the array of multiple alleles at a single locus. Gametes are extremely unstable. Mutation now embraces point mutation, chromosomal aberrations, recombination, and chromosomal segregation.

Finally, individuals comprise a single array, analogous to a multiple allele series at a single locus. As a unit of heredity, the individual is clearly the least stable, as mutation now includes all factors listed for gametes plus variation which is generated by the union of gametes.



In summary, then, it is clear that there are different levels of genetic organization and that the hereditary units for each level are amenable to genetic analysis. The gene locus is an organized chromosomal segment which contains many different mutational and recombinational sites. It is in some respects, comparable to a minute chromosome. The chromosome is composed of a collection of gene loci. Since it is a morphological entity, there is no ambiguity in its definition. The gamete is a collection of chromosomes such that one chromosome of each homologous set is included. Finally, the diploid individual is a combination of two gametes.

(b) *Characterization of a Random-mating Population by Different Units of Inheritance*

A random-mating system is one in which the frequencies of matings between the various classes of genotypes are equal to the product of the frequencies of the genotypes themselves. A random-mating population in equilibrium is one in which the frequencies of the genotypes do not change from one generation to the next. Thus, if the genotypic array of a population is  $\sum f_i G_i$ , where  $G_i$  is the  $i$ th genotype and  $f_i$  its frequency, then the system of random matings may be generated by squaring this array, i.e.

$$(\sum f_i G_i)^2 = \sum_{ij} f_i f_j G_i G_j,$$

where  $G_i G_j$  represents the mating between the  $i$ th and  $j$ th genotypes. The results of these matings produces another population, the array of which may be denoted as  $\sum f'_i G_i$ . If  $f'_i = f_i$ , then the population is said to be in equilibrium.

It is now useful to consider how a representation of a random-mating population in equilibrium can be generated by the various basic units of heredity, i.e. genes, chromosomes, gametes, and individuals. The basic principle is that an equilibrium population for genotypes at a single locus may be obtained by "squaring" the array of multiple alleles for the locus. This, of course, assumes that the allelic array has been derived from the equilibrium population without selection. This principle holds for any unit of inheritance and for any system of linkages. Thus, if  $\sum f_i H_i$  represents the array of multiple alleles at a single locus for any system of hereditary units, the equilibrium population of genotypes at the locus may be generated as

$$(\sum f_i H_i)^2 = \sum_{ij} f_i f_j H_i H_j.$$

If the units of inheritance are diploid genotypes, then the combination  $H_i H_j$  represents the full-sib array which results from the mating of the  $i$ th and the  $j$ th genotypes.

If genes or chromosomes are considered as units of inheritance then more than one locus must eventually be considered. In this case, let  $\sum f_i^r H_i^r$  represent the array of alleles at the  $r$ th locus. The equilibrium population for all loci is then obtained by multiplying together the genotypic arrays produced at each locus, i.e.

$$\prod_{r=1}^n [\sum_i f_i^r H_i^r]^2.$$



Confining attention now to a single locus (for any hereditary unit), it is convenient to represent the operation of squaring by forming a two-way multiplication table. Thus  $(\sum f_i H_i)^2$  can be set out as follows:

	$H_1$ ( $f_1$ )	$H_2$ ( $f_2$ )	$\dots$	$H_m$ ( $f_m$ )
$H_1$ ( $f_1$ )	$H_1 H_1$ ( $f_1$ ) <sup>2</sup>	$H_1 H_2$ ( $f_1 f_2$ )	$\dots$	$H_1 H_m$ ( $f_1 f_m$ )
.	.	.	.	.
.	.	.	.	.
.	.	.	.	.
$H_m$ ( $f_m$ )	$H_m H_1$ ( $f_m f_1$ )	$H_m H_2$ ( $f_m f_2$ )	$\dots$	$H_m H_m$ ( $f_m$ ) <sup>2</sup>

The usefulness of this representation is that it provides a pattern which is clearly amenable to all of the ramifications of the analysis of variance for a two-way classification with proportional subclass frequencies. Therefore, effects and interactions can be defined and a mathematical model constructed from an identity of means. Also an identity in sums of squares can be used to define an orthogonal partitioning of the total variance into component parts associated with the various classes of effects.

Fisher (1918) laid the foundations for such an analysis using "genes" as the hereditary units. This early pioneering work has been extended by Kempthorne (1957, for general reference). However, it is obvious that this Fisher-Kempthorne approach can be generalized to include any level of hereditary unit.

### (c) Definitions

Referring, now, to the two-way table, generalized definitions can be made as follows:

Let

$H_i$  = hereditary unit (gene, chromosome, gamete, or individual),

$f_i$  = frequency of  $H_i$ ,

$h_{ij}$  = genotypic deviation associated with the  $H_i H_j$  combination, such that  $\sum_{ij} f_i f_j h_{ij} = 0$ .

Any genotypic value,  $h_{ij}$ , may be represented by the following identity,

$$h_{ij} \equiv h_{i.} + h_{.j} + (h_{ij} - h_{i.} - h_{.j}),$$

which may be recast as

$$h_{ij} = \gamma_i + \gamma_j + \theta_{ij},$$

where

$$\gamma_i = \sum_j f_j h_{ij} = \text{general combining ability (g.c.a.) effect of } H_i,$$

$$\gamma_j = \sum_i f_i h_{ij} = \text{g.c.a. effect of } H_j,$$

and

$$\theta_{ij} = h_{ij} - h_{i.} - h_{.j} = \text{specific combining ability (s.c.a.) effect for the combination } H_i H_j.$$

The total variance may be partitioned as follows:

$$\sum_{ij} f_i f_j h_{ij}^2 \equiv 2 \sum_i f_i \gamma_i^2 + \sum_{ij} f_i f_j \theta_{ij}^2,$$

which may be recast as

$$\sigma_H^2 = \sigma_{\text{g.c.a.}}^2 + \sigma_{\text{s.c.a.}}^2,$$

where

$$\sigma_{\text{g.c.a.}}^2 = \text{g.c.a. variance,}$$

and

$$\sigma_{\text{s.c.a.}}^2 = \text{s.c.a. variance.}$$

The usual analysis based on genes may be regarded as a special case of the above more general representation. If  $H_i$  ( $i = 1, \dots, m$ ) are genes (i.e. multiple alleles at a single locus), then the symbolization may be converted to the more usual gene notation as follows:

$$\gamma_k = \alpha_k = \text{additive effect of } A_k,$$

$$\theta_{ij} = \delta_{ij} = \text{non-additive (dominance) effect of } A_i A_j,$$

$$\sigma_{\text{g.c.a.}}^2 = \sigma_A^2 = \text{additive genetic variance,}$$

and

$$\sigma_{\text{s.c.a.}}^2 = \sigma_D^2 = \text{non-additive (dominance) variance.}$$

It may be noted that the terms general and specific combining ability effects and variances are introduced to apply to all units of inheritance and in the case of genes they are synonymous with the well-established terms, additive and non-additive effects and variances. This has been done to avoid confusion which would possibly otherwise exist if one were to apply the words additive and non-additive to effects and variances of higher units than the gene. For example, in the case where genotypes involve genes at more than one gene locus, the g.c.a. effects of higher units than the gene are a function not only of additive but also of certain non-additive gene effects.

In the more generalized argument of this study, and others to follow, it is convenient to use the individual as the unit of inheritance and to use certain covariances between relatives as the genetic parameters of particular interest.

The following defines three covariances and relates them to the "individual" general and specific combining ability variances.

To characterize the random-mating population with the individual as the unit of inheritance, let  $\Sigma f_i H_i$  designate the array of parent individuals undergoing random mating, and let  $\Sigma f_i h_i = 0$  denote the corresponding array of genotypic values. The cross between the  $i$ th and the  $j$ th parents yields a full-sib array the mean of which may be designated as  $h_{ij} = \sum_k p_{ijk} h_{ijk}$ . Note that the  $ijk$ th progeny has an arbitrary frequency  $p_{ijk}$  (such that  $\sum_k p_{ijk} = 1$ ), and hence any system of linkage may occur.

The full-sib means may be set out in the usual two-way table as follows:

	$H_1$ ( $f_1$ )	$H_2$ ( $f_2$ )	$H_m$ ( $f_m$ )	
$H_1$ ( $f_1$ )	$h_{11}$ ( $f_1$ ) <sup>2</sup>	$h_{12}$ ( $f_1 f_2$ )	$h_{1m}$ ( $f_1 f_m$ )	$h_{1..}$
.	.	.	.	.
.	.	.	.	.
.	.	.	.	.
$H_m$ ( $f_m$ )	$h_{m1}$ ( $f_m f_1$ )	$h_{m2}$ ( $f_m f_2$ )	$h_{mm}$ ( $f_m$ ) <sup>2</sup>	$h_{m..}$
				$h_{...} = 0$

The parent-offspring covariance (designated as Cov(PO)) may be defined as the expected value of the cross-product of the genotypic deviation of an arbitrary parent individual and the mean genotypic deviation of the half-sib array associated with the parent individual. If  $h_i$  is the genotypic deviation of the parent genotype  $H_i$ , then

$$\begin{aligned}\text{Cov(PO)} &= E(h_i \cdot h_{i..}) \\ &= \Sigma f_i h_i \cdot h_{i..}\end{aligned}$$

The covariance of full-sibs (designated as Cov(FS)) may be defined as the expected value of the cross-product of the genotypic deviation of an arbitrary individual and the mean genotypic deviation of the full-sib array of which it is a member. Thus,

$$\begin{aligned}\text{Cov(FS)} &= E(h_{ijk} \cdot h_{ij.}) \\ &= \Sigma_{ij} f_i f_j \Sigma_k p_{ijk} h_{ijk} \cdot h_{ij.} \\ &= \Sigma_{ij} f_i f_j (h_{ij.})^2.\end{aligned}$$

The covariance of half-sibs (designated as  $\text{Cov}(\text{HS})$ ) may be defined as the expected value of the cross-product of the genotypic deviation of an arbitrary individual and the mean genotypic deviation of the half-sib array of which it is a member. Thus

$$\begin{aligned}\text{Cov}(\text{HS}) &= E(h_{ijk} \cdot h_{i..}) \\ &= \sum_{ij} f_i f_j \sum_k p_{ijk} h_{ijk} \cdot h_{i..} \\ &= \sum f_i (h_{i..})^2.\end{aligned}$$

Interpretation of the general and specific combining ability variance components may be made in terms of covariances of full-sibs and half-sibs as follows:

$$\begin{aligned}\sigma_H^2 &= \sum_{ij} f_i f_j (h_{ij.})^2 \\ &= \text{Cov}(\text{FS}), \\ \sigma_{\text{g.c.a.}}^2 &= 2 \sum f_i (\gamma_i)^2 \\ &= 2 \sum f_i (h_{i..})^2 \\ &= 2 \text{Cov}(\text{HS}),\end{aligned}$$

and

$$\begin{aligned}\sigma_{\text{s.c.a.}}^2 &= \sum_{ij} f_i f_j \theta_{ij}^2 \\ &= \text{Cov}(\text{FS}) - 2 \text{Cov}(\text{HS}).\end{aligned}$$

The above definitions apply to a completely general genetic situation. In terms of genes this means a genetic situation which is completely arbitrary for (i) number of alleles at any locus, (ii) number of loci, (iii) system of linkages, and (iv) dominance and epistatic relationships.

It may be noted that not all of the above definitions are used in the present paper. However, this study is the first of a series which will include a treatment of full-sib and half-sib selection, and, collectively, these studies will make use of the entire range of definitions. Hence, in this first paper, it seems worth while to completely set out the notion of higher-order units of inheritance and their associated definitions.

## II. CONSEQUENCES OF TRUNCATION SELECTION BASED ON INDIVIDUAL PHENOTYPE

This study is concerned with individual selection within the framework of a breeding programme which consists of cycles. In each cycle, selection is applied to members of a random-mating population and the selected individuals are then mated at random to provide the population for the next cycle. The original unselected population, with which the selection programme starts, is assumed to be in equilibrium.

The arguments to be developed are formulated for an idealized situation in which the populations are infinite in size and, therefore, can be characterized by continuous distributions. The method of defining selection values is that due to



Kimura (1958) and some parts of the more generalized argument are found in the work of Kimura (1958).

In the remainder of the paper the consequences of selection are examined for three levels of complexity in terms of gene structure: (i) a single locus level, (ii) a level involving two linked loci, and (iii) a generalized level. These studies, fundamentally, represent three different approaches to the problem of describing the response to selection by utilizing different units of inheritance. In the first analysis, the gene is considered as the basic unit and the consequences are examined when selection is applied to genotypes at a single gene locus. The total response is then obtained by summing over all gene loci. The gamete is considered as the basic unit in the second analysis with, however, a gene interpretation so that the detailed results can be compared with those of the gene analysis.

Finally, in the last section, generalizations are made by using analyses in which the individual is considered as the basic unit of inheritance. However, the frequency of an individual is necessarily specified in terms of the frequencies of the gametes which united to form the individual. In order to compare the results with the previous analyses, the generalized results are interpreted in terms of the example consisting of two loci which are linked.

(a) *Selection of Genotypes Generated by Alleles at One Locus*

(i) *Definitions*

(1) *Parameters of the Random-mating Population in Equilibrium.*—The random-mating population generated by  $m$  alleles at one locus may be set out in the following two-way table:

	$A_1$ ( $p_1$ )	$A_2$ ( $p_2$ )	$A_m$ ( $p_m$ )	
$A_1$ ( $p_1$ )	$d_{11}$ ( $p_1$ ) <sup>2</sup> $w_{11}$	$d_{12}$ ( $p_1 p_2$ ) $w_{12}$	$d_{1m}$ ( $p_1 p_m$ ) $w_{1m}$	$\alpha_1$
.	.	.	.	.
.	.	.	.	.
.	.	.	.	.
$A_m$ ( $p_m$ )	$d_{m1}$ ( $p_m p_1$ ) $w_{m1}$	$d_{m2}$ ( $p_m p_2$ ) $w_{m2}$	$d_{mm}$ ( $p_m$ ) <sup>2</sup> $w_{mm}$	$\alpha_m$
				0

In this table,

(a)  $A_1, A_2, \dots, A_m$  represent the  $m$  alleles,

(b)  $p_i$  = frequency of  $A_i$ ,

(c)  $d_{ij}$  = genotypic value of  $A_iA_j$ , such that

$$\mu_0 = \sum_{ij} p_i p_j d_{ij} = 0,$$

and

(d)  $w_{ij}$  = selection value of  $A_iA_j$  (defined later).

The genotypic value,  $d_{ij}$ , may be characterized by the following gene model,

$$d_{ij} = \alpha_i + \alpha_j + \delta_{ij},$$

where

$$\alpha_i = \sum_j p_j d_{ij} = \text{additive effect of the } A_i \text{ allele,}$$

and

$$\delta_{ij} = d_{ij} - \alpha_i - \alpha_j = \text{dominance effect associated with } A_iA_j.$$

The total genotypic variance may be partitioned as follows:

$$\sum_{ij} p_i p_j d_{ij}^2 = 2 \sum_i p_i \alpha_i^2 + \sum_{ij} p_i p_j \delta_{ij}^2,$$

which may be represented symbolically as

$$\sigma_G^2 = \sigma_A^2 + \sigma_D^2,$$

where

$$\sigma_G^2 = \sum_{ij} p_i p_j d_{ij}^2 = \text{genotypic variance,}$$

$$\sigma_A^2 = 2 \sum_i p_i \alpha_i^2 = \text{additive genetic variance,}$$

and

$$\sigma_D^2 = \sum_{ij} p_i p_j \delta_{ij}^2 = \text{dominance variance.}$$

(2) *Selection Values.*—The assumptions in the following argument may be listed as follows:

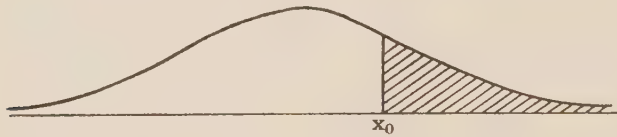
- (i) The genotypic variability of the characteristic which is being studied is controlled by genes, each of small effect, at many loci.
- (ii) The phenotypic variability, due to environmental effects together with that due to the segregation at the numerous loci, is normally distributed with mean zero and variance  $\sigma^2$ .
- (iii) The initial random-mating population is in equilibrium for genes at all loci.

With regard to the particular locus in question, it is conceptually possible to subdivide the entire populations of individuals into groups corresponding to the various genotypes  $A_iA_j$  ( $i, j = 1, \dots, m$ ). The relative frequencies of these groups are  $p_i p_j$  ( $i, j = 1, \dots, m$ ). From the above assumptions, it is clear that the subpopulation of individuals having a given genotype,  $A_iA_j$ , is normally distributed with genotypic mean,  $d_{ij}$ , and variance,  $\sigma_{ij}^2$ . That is to say, different members of

this population all have the genotype  $A_iA_j$ , for the locus under study, but may have different genes for the other loci, as well as different environmental conditions. These differences generate the normal population with genotypic mean,  $d_{ij}$ , and variance,  $\sigma_{ij}^2$ . With regard to the definition of selection values, there are two further assumptions which need to be made concerning the magnitudes of  $d_{ij}$  and  $\sigma_{ij}^2$ . These are: (i)  $d_{ij}$  is small in relation to  $\sigma$  so that the quantities  $(d_{ij}/\sigma)^2$  and  $(d_{ij}/\sigma^2)^2$  may be neglected, and (ii) the genotypic variance generated by the locus in question is small, relative to the total phenotypic variance, so that  $\sigma_{ij}^2 \cong \sigma^2$ .

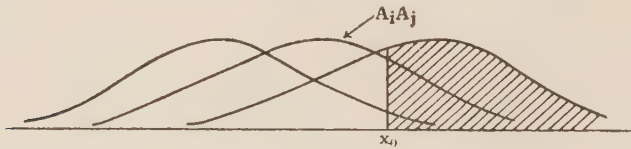
The "truncation" selection programme is such that all members of the entire population which have a greater phenotypic value than a given value, say  $x_0$ , will be selected for mating at random to produce the next generation of individuals. This selection programme may be diagrammed as follows:

- (i) Selection programme depicted for the entire population:



Individuals represented by shaded area are mated at random to produce the next population.

- (ii) Selection programme depicted in terms of subpopulations:



The selection value,  $w_{ij}$ , for the genotype  $A_iA_j$  is defined to be proportional to the probability that an individual of the genotype  $A_iA_j$  survives selection. This probability is

$$\begin{aligned}
 \Pr(x > x_0) &= \frac{1}{\sigma_{ij}\sqrt{(2\pi)}} \int_{x_0}^{\infty} \exp\{-(x-d_{ij})^2/2\sigma_{ij}^2\} dx \\
 &\cong \frac{1}{\sigma\sqrt{(2\pi)}} \int_{x_0}^{\infty} \exp\{-(x^2-2xd_{ij}+d_{ij}^2)/2\sigma^2\} dx \\
 &\cong \frac{1}{\sigma\sqrt{(2\pi)}} \int_{x_0}^{\infty} \exp[-(x^2/2\sigma^2)] \cdot \exp(xd_{ij}/\sigma^2) dx \\
 &\cong \frac{1}{\sigma\sqrt{(2\pi)}} \int_{x_0}^{\infty} \exp[-(x^2/2\sigma^2)][1+(xd_{ij}/\sigma^2)] dx
 \end{aligned}$$

$$\begin{aligned}
&\cong \frac{1}{\sigma\sqrt{(2\pi)}} \int_{x_0}^{\infty} \exp[-(x^2/2\sigma^2)] dx + (d_{ij}/\sigma^2) \cdot \frac{1}{\sigma\sqrt{(2\pi)}} \int_{x_0}^{\infty} x \cdot \exp[-(x^2/2\sigma^2)] dx \\
&= v + (d_{ij}/\sigma^2) i v \\
&\propto 1 + (d_{ij}/\sigma^2) i,
\end{aligned}$$

where  $v$  is the proportion of the original population which is selected and  $i$  is the selection differential, i.e.

$$i = \left\{ \frac{1}{\sigma\sqrt{(2\pi)}} \int_{x_0}^{\infty} x \cdot \exp[-(x^2/2\sigma^2)] dx \right\} / v.$$

The selection value for  $A_i A_j$  is then defined to be

$$w_{ij} = 1 + (i/\sigma^2) d_{ij},$$

or

$$w_{ij} = 1 + \bar{i}(d_{ij}/\sigma),$$

where  $\bar{i} = i/\sigma =$  standardized form of the selection differential.

## (ii) *Consequences of One Cycle of Selection*

The problem for review in this section is that of determining the change in population parameters due to a single cycle of truncation selection. The cycle starts with a random-mating population in equilibrium as described in the previous section. This population is designated as  $\Pi_0$  and the parameters associated with this population are designated with the superscript "0". The progeny population produced by the first cycle of selection is designated as  $\Pi_1$ , and its parameters are designated with superscript "1". The elements and variances of the gene model are always assumed to be those associated with  $\Pi_0$ .

The frequency of  $A_i A_j$  following selection is

$$p_i^0 p_j^0 w_{ij}^0 = p_i^0 p_j^0 + (i/\sigma^2) p_i^0 p_j^0 d_{ij}^0.$$

The total frequency of all selected genotypes is

$$\sum_{ij} [p_i^0 p_j^0 + (i/\sigma^2) p_i^0 p_j^0 d_{ij}^0] = 1.$$

The genotypic mean of the selected parents is

$$\begin{aligned}
\mu_s &= \sum_{ij} [p_i^0 p_j^0 + (i/\sigma^2) p_i^0 p_j^0 d_{ij}^0] d_{ij}^0 \\
&= i(\sigma_G^2/\sigma^2) \\
&= i(\text{heritability in the broad sense}).
\end{aligned}$$

The gene frequency for the  $A_i$  allele in the selected population is

$$\begin{aligned}
p_i^1 &= \sum_j [p_i^0 p_j^0 + (i/\sigma^2) p_i^0 p_j^0 d_{ij}^0] \\
&= p_i^0 + \bar{i} p_i^0 (\alpha_i/\sigma) \\
&= p_i^0 + \Delta p_i.
\end{aligned}$$



The total gene array produced by the selected parents is

$$\sum p_i^1 A_i = \sum (p_i^0 + \Delta p_i) A_i.$$

Hence, the progeny genotypic array which results from random mating the selected parents is obtained by squaring the gene array as follows:

$$(\sum p_i^1 A_i)^2 = \sum_{ij} p_i^0 p_j^0 A_i A_j + (i/\sigma) \sum_{ij} p_i^0 p_j^0 (\alpha_i + \alpha_j) A_i A_j + i^2 \sum_{ij} p_i^0 p_j^0 (\alpha_i/\sigma)(\alpha_j/\sigma) A_i A_j.$$

The progeny mean is obtained by substituting  $d_{ij}^0$  for  $A_i A_j$ . Thus

$$\mu_1 = (i/\sigma) \sigma_A^2 + i^2 \sum_{ij} p_i^0 p_j^0 (\alpha_i/\sigma)(\alpha_j/\sigma) \delta_{ij}.$$

This last equation can be simplified by assuming that  $(\alpha/\sigma)$  is small so that the square or product of two such quantities can be neglected. Assumptions of this sort will be made throughout this study. In this case

$$\mu_1 = i(\sigma_A^2/\sigma^2).$$

For a single locus, the increment advance in the genotypic mean due to selection is equal to the difference between the genotypic means of  $\Pi_1$  and  $\Pi_0$ , i.e.

$$\begin{aligned} \Delta\mu_{10} &= \mu_1 - \mu_0 \\ &= i(\sigma_A^2/\sigma^2) \\ &= i(\text{heritability in the narrow sense}). \end{aligned}$$

For individual loci these quantities are negligible, but the sum of such effects over a large number of loci is assumed to be appreciable.

This increment change in means may be predicted from the difference between the phenotypic means (which are equal to genotypic means when infinite populations are considered) of the selected parents and the unselected original population. This difference is defined to be the value  $i$ . The prediction equation is

$$\Delta\mu_{10} = Gi.$$

Hence  $G = \sigma_A^2/\sigma^2 = \text{heritability in the narrow sense}$ .

Thus, prediction of the genotypic advance can be made for the case of a single cycle of selection operating in a genetic system composed of an arbitrary number of alleles at a single locus.

If the cycle of selection is followed by random mating without selection (either artificial or natural) the population structure remains unchanged and the population mean remains at  $\mu = i(\sigma_A^2/\sigma^2)$ , i.e. the population is in equilibrium. This assumes that the gene has negligible mutation rate in relation to the time span concerned. That is, point mutation together with recombination within a locus yield a low total mutation rate.

(iii) *Consequences of Two or More Cycles of Continuous Selection*

The problem in this section is to describe the changes in parameters which occur with two or more cycles of continuous selection.\*

In the previous section it was shown that the progeny resulting from the first cycle of selection constitutes a random-mating population in equilibrium which may be characterized as

$$\begin{aligned}\Pi_1 &= \sum_{ij} p_i^1 p_j^1 A_i A_j, \\ \text{with mean} \quad \mu_1 &= \sum_{ij} p_i^1 p_j^1 d_{ij}^0 \\ &= i(\sigma_A^2/\sigma^2).\end{aligned}$$

Assuming that the selection differential  $i$  remains the same and that the phenotypic variance is essentially the same as in  $\Pi_0$ , the frequency of  $A_i A_j$  following selection in  $\Pi_1$  is

$$\begin{aligned}p_i^1 p_j^1 w_{ij}^1 &= p_i^1 p_j^1 [1 + (i/\sigma) d_{ij}^1] \\ &= p_i^1 p_j^1 [1 + (i/\sigma)(d_{ij}^0 - \mu_1)] \\ &= p_i^1 p_j^1 [1 + (i/\sigma) d_{ij}^0 - (i/\sigma)(i \sigma_A^2/\sigma^2)] \\ &= p_i^1 p_j^1 \{1 + (i/\sigma) d_{ij}^0 - i^2 [2 \sum p_i^0 (\alpha_i/\sigma)^2]\} \\ &\cong p_i^1 p_j^1 \{1 + (i/\sigma) d_{ij}^0\}.\end{aligned}$$

It may be noted that when more than one locus is considered, the relation

$$d_{ij}^1 = d_{ij}^0 - i(\sigma_A^2/\sigma^2),$$

(where  $\sigma_A^2$  is the additive variance for the particular locus in question) still holds. The sum of these frequencies is

$$\sum_{ij} p_i^1 p_j^1 [1 + (i/\sigma) d_{ij}^0] = 1.$$

The frequency of  $A_i$  becomes

$$\begin{aligned}p_i^2 &= \sum_j p_i^1 p_j^1 [1 + (i/\sigma) d_{ij}^0] \\ &\cong p_i^0 [1 + 2i(\alpha_i/\sigma)].\end{aligned}$$

Hence

$$\begin{aligned}\Pi_2 &= (\sum p_i^2 A_i)^2 \\ &= \sum_{ij} p_i^2 p_j^2 A_i A_j,\end{aligned}$$

and

$$\begin{aligned}\mu_2 &= \sum_{ij} p_i^2 p_j^2 d_{ij}^0 \\ &\cong 2i(\sigma_A^2/\sigma^2).\end{aligned}$$

\* The phrase "continuous selection" means that selection occurs in each and every cycle under consideration.

The total increment change in the population mean due to the two cycles of selection is then

$$\begin{aligned}\Delta\mu_{20} &= \mu_2 - \mu_0 \\ &\cong 2i(\sigma_A^2/\sigma^2).\end{aligned}$$

More generally, after  $n$  generations of continuous selection,

$$p_i^n \cong p_i^0 + n i p_i^0 (\alpha/\sigma),$$

and

$$\Delta\mu_{n0} \cong n i (\sigma_A^2/\sigma^2).$$

These responses refer to the responses due to artificial selection unopposed by natural selection. This assumption will always be made unless stated otherwise.

It is clear from these results that the *predicted* response to selection is linear when plotted against the number of selection cycles. Actually, however, the response to selection results in an asymptotic approach to the goal of selection whether it is that of homozygosity or a stable equilibrium. Hence the basis of prediction becomes more subject to error as the mean of the selected population becomes farther removed from its original position. This is primarily due to the increasing magnitude of the neglected quantities in the analysis. Thus, starting with the basic assumption that the square and products of the quantities  $(\alpha/\sigma)$  are negligible, it is assumed that

$$\begin{aligned}(i/\sigma)d_{ij}^n &= (i/\sigma)(d_{ij}^0 - \mu_n) \\ &\cong (i/\sigma)d_{ij}^0,\end{aligned}$$

that is

$$(i/\sigma)\mu_n \cong 0,$$

where  $\mu_n$  is the increment change in the population mean due to  $n$  cycles of selection operating on the single locus. Clearly, as  $n$  increases this approximation becomes worse. Hence the linear predicted response holds for only a relatively short segment of the total response curve.

A general discussion of the attainment of the selection goal follows in the next section.

#### (iv) *Attainment of Selection Goal*

Selection operating on individuals which are subject to random mating leads to either a homozygous population or to a polymorphic population in equilibrium. The following discusses the attainment of either one of the two possible selection goals for the situation of constant selection values applied to genotypes generated by only two alleles at a single locus. In this case selection results in a stable polymorphic equilibrium if overdominance exists, and if overdominance does not exist selection results in a population homozygous for the most desirable allele. A more detailed argument follows.

Assume that as a result of selection the ratio of the three genotypes surviving selection is

$$A_1A_1 : A_1A_2 : A_2A_2 = (1-s_1) : 1 : (1-s_2) = (w_{11}/w_{12}) : 1 : (w_{22}/w_{12}).$$

Clearly then

$$s_1 \cong -(i/\sigma^2)(d_{11}-d_{12}) = -(i/\sigma^2)[(\alpha_1-\alpha_2)+(\delta_{11}-\delta_{12})],$$

and

$$s_2 \cong -(i/\sigma^2)(d_{22}-d_{12}) = -(i/\sigma^2)[(\alpha_2-\alpha_1)+(\delta_{22}-\delta_{12})].$$

From these equations it appears that the selection coefficients, and hence the selection values, are a function of both additive and non-additive effects.

The increment changes in gene frequencies, as before, are

$$\Delta p_1 \cong (i/\sigma^2)p_1\alpha_1,$$

and

$$\Delta p_2 \cong (i/\sigma^2)p_2\alpha_2.$$

Thus, changes in gene frequencies are functions of only additive gene effects.

It is obvious that the values for  $\alpha$ 's and  $\delta$ 's change slightly with every cycle of selection. As the goal of selection is approached,

$$\Delta p_i \rightarrow 0,$$

and

$$\alpha's \rightarrow 0,$$

$$\sigma_A^2 \rightarrow 0.$$

The specific goal of selection depends on the degree of dominance:

- (1) If partial or no dominance exists, and if  $A_1$  is the most favoured allele, then  $s_1$  is negative and  $s_2$  is positive, and, therefore, the order of selection preference is  $A_1A_1 > A_1A_2 > A_2A_2$ . The changes in gene frequencies are:  $\Delta p_1 > 0$  and  $\Delta p_2 < 0$  for all values of  $p_1$  and  $p_2$ . Thus, the population tends to homozygosity of the  $A_1A_1$  genotype.
- (2) If overdominance exists, both  $s_1$  and  $s_2$  are positive, irrespective of gene frequencies. Therefore, the heterozygote will be preferred over both homozygotes at all times. A stable equilibrium is reached when the gene frequencies become

$$p_1 = s_2/(s_1+s_2),$$

and

$$p_2 = s_1/(s_1+s_2).$$

Hence, the increment change in gene frequency of the  $A_1$  allele will be positive or negative depending on the value of  $p_1$  in the population undergoing selection relative to the equilibrium frequency of  $p_1$ .

The trend to the selection goal can be examined from the point of view of the change in the genotypic mean of the population due to selection. The increment change for a cycle of selection has been shown to be

$$\Delta\mu = i(\sigma_A^2/\sigma^2).$$



Since  $\sigma_A^2$  is necessarily positive or zero, selection continuously increases the population mean until the maximum mean value is attained. This value occurs when  $\Delta\mu = 0$ , i.e. when  $\sigma_A^2 = 0$ . This is true for any degree of dominance. Thus, when partial dominance exists, the population mean is a maximum when the population is homozygous for  $A_1A_1$ , and it is only at this composition of the population that  $\sigma_A^2 = 0$  and  $\Delta\mu = 0$ . When overdominance exists  $\sigma_A^2 = 0$  and, hence,  $\Delta\mu = 0$  when the stable equilibrium is reached. At this point the population has a maximum value.

When more than two alleles are considered, the equilibrium conditions are more complicated. All that needs to be mentioned in this study is that selection again may result in either a homozygous or a polymorphic population. The necessary and sufficient conditions for the maintenance of all alleles in a stable equilibrium have been given by Owen (1953), Kimura (1956), and Mandel (1959).

(b) *Selection of Genotypes Generated by Alleles at Two Loci which may be Linked*

This section is concerned with the consequences of selection which operates on genotypes generated by an arbitrary number of alleles at each of two linked loci. The argument holds for any recombination value and for arbitrary sets of dominance and epistatic values.

(i) *Definitions.*—In extending the considerations from one to two loci, it is useful to employ the *gamete* as the unit of inheritance rather than the gene. However, an interpretation of the gametic analysis will be made in terms of gene effects and variances.

With more than one locus to consider, the notation necessarily becomes more complicated since both loci and genes need to be identifiable. For convenience, the Kempthorne (1957) notation is used. Let  $\sum_{i=1}^m p_i^1 A_i^1$  represent the array of alleles at the first locus and  $\sum_{k=1}^n p_k^2 A_k^2$  represent the array of alleles at the second locus. The two loci may exhibit linkage with a recombination frequency whose value is  $y$ . Let the frequency of the gamete  $(A_i^1 A_k^2)$  be  $f_{ik} = p_i^1 p_k^2$ . The gametic array is then  $\sum_{ik} f_{ik} (A_i^1 A_k^2)$ .

The random-mating population in equilibrium may be generated by squaring the gametic array, i.e.

$$[\sum_{ik} f_{ik} (A_i^1 A_k^2)]^2 = \sum_{ijkl} f_{ik} f_{jl} (A_i^1 A_k^2) (A_j^1 A_l^2).$$

This representation is set out in the following two-way table in which the genotypic value of  $(A_i^1 A_k^2) (A_j^1 A_l^2)$  is  $d_{ik,jl}$  whose value is chosen such that

$$\sum_{ijkl} f_{ik} f_{jl} d_{ik,jl} = 0.$$

	$A_1^1 A_1^2$ ( $f_{11}$ )	$A_1^1 A_2^2$ ( $f_{12}$ )	$A_m^1 A_n^2$ ( $f_{mn}$ )
$A_1^1 A_1^2$ ( $f_{11}$ )	$d_{11.11}$ ( $f_{11}$ ) <sup>2</sup> $w_{11.11}$	$d_{11.12}$ ( $f_{11}f_{12}$ ) $w_{11.12}$	$d_{11.mn}$ ( $f_{11}f_{mn}$ ) $w_{11.mn}$
.	.	.	.
.	.	.	.
.	.	.	.
$A_m^1 A_n^2$ ( $f_{mn}$ )	$d_{mn.11}$ ( $f_{mn}f_{11}$ ) $w_{mn.11}$	$d_{mn.12}$ ( $f_{mn}f_{12}$ ) $w_{mn.12}$	$d_{mn.mn}$ ( $f_{mn}$ ) <sup>2</sup> $w_{mn.mn}$

The genotypic value,  $d_{ik.jl}$ , is characterized by the Kempthorne gene model as follows:

$$d_{ik.jl} = \alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2 + \delta_{ij}^1 + \delta_{kl}^2 + (\alpha\alpha)_{ik} + (\alpha\alpha)_{il} + (\alpha\alpha)_{jk} + (\alpha\alpha)_{jl} \\ + (\alpha\delta)_{ikl} + (\alpha\delta)_{jkl} + (\delta\alpha)_{ijk} + (\delta\alpha)_{ijl} + (\delta\delta)_{ijkl},$$

where

$\alpha_u^a$  = additive genetic effect of the  $A_u^a$  allele,

$\delta_{uv}^a$  = dominance effect for the  $A_u^a A_v^a$  genotype,

$(\alpha\alpha)_{ik}$  = additive  $\times$  additive epistatic effect associated with genes  $A_i^1$  and  $A_k^2$ ,

$(\alpha\delta)_{ikl}$  = additive  $\times$  dominance epistatic effect associated with the gene  $A_i^1$  and the genotype  $A_k^2 A_l^2$ , and

$(\delta\delta)_{ijkl}$  = dominance  $\times$  dominance epistatic effect associated with the genotypes  $A_i^1 A_j^1$  and  $A_k^2 A_l^2$ .

The total genotypic variance may be partitioned as

$$\sigma_G^2 = \sigma_A^2 + \sigma_D^2 + \sigma_{AA}^2 + \sigma_{AD}^2 + \sigma_{DD}^2,$$

where

$\sigma_G^2$  = total genotypic variance generated by the two loci,

$\sigma_A^2$  = additive genetic variance,

$\sigma_D^2$  = dominance variance,

$\sigma_{AA}^2$  = additive  $\times$  additive variance,

$\sigma_{AD}^2$  = additive  $\times$  dominance variance,

and

$\sigma_{DD}^2$  = dominance  $\times$  dominance variance.

The selection value  $w_{ik,jl}$  for the genotype  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  is defined to be proportional to the probability that an individual of the genotype  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  survives selection. Following the same argument as given for the single locus case, the selection value is

$$w_{ik,jl} = 1 + (i/\sigma^2)d_{ik,jl}.$$

(ii) *Consequences of One Cycle of Selection.*—The objective of this section is to determine the changes in population parameters due to one cycle of truncation selection which is then followed by an indefinite number of cycles of random mating without selection. The programme starts with a random-mating population in equilibrium, which is designated as  $\Pi_0$ . As before, to simplify notation all parameters of the gene model are assumed to be those associated with  $\Pi_0$  and therefore will not have a superscript notation.

The frequency of the genotype  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  following selection is

$$f_{ik,jl}^0 w_{ik,jl}^0 = f_{ik,jl}^0 [1 + (i/\sigma^2)d_{ik,jl}^0].$$

The total frequency over all selected genotypes is

$$\sum_{ijkl} f_{ik,jl}^0 f_{jl}^0 [1 + (i/\sigma^2)d_{ik,jl}^0] = 1.$$

The genotypic mean of the selected parents is

$$\mu_s = \sum_{ijkl} f_{ik,jl}^0 f_{jl}^0 [1 + (i/\sigma^2)d_{ik,jl}^0] d_{ik,jl}^0.$$

The progeny population resulting from random mating the selected parents can be generated by squaring the gametic array produced by the selected parents. To obtain this array it is first necessary to consider the gametic array produced by an arbitrary genotype,  $(A_i^1 A_k^2)(A_j^1 A_l^2)$ , which is

$$\{(1-y)/2\}(A_i^1 A_k^2 + A_j^1 A_l^2) + (y/2)(A_i^1 A_l^2 + A_j^1 A_k^2)\}.$$

The gametic array for all selected parents is then,

$$\sum_{ijkl} f_{ik,jl}^0 w_{ik,jl}^0 \{[(1-y)/2](A_i^1 A_k^2 + A_j^1 A_l^2) + (y/2)(A_i^1 A_l^2 + A_j^1 A_k^2)\},$$

which, on using a summation device illustrated by Kempthorne (1957), is equal to

$$\begin{aligned} & \sum_{ijkl} \{f_{ik,jl}^0 w_{ik,jl}^0 (1-y)/2 + f_{jl,ik}^0 w_{jl,ik}^0 (1-y)/2 + f_{il,jk}^0 w_{il,jk}^0 (y/2) + f_{jk,il}^0 w_{jk,il}^0 (y/2)\} (A_i^1 A_k^2) \\ &= \sum_{ijkl} \{f_{ik,jl}^0 w_{ik,jl}^0 (1-y) + f_{il,jk}^0 w_{il,jk}^0 (y)\} (A_i^1 A_k^2) \\ &= \sum_{ik} f_{ik}^1 (A_i^1 A_k^2), \end{aligned}$$

where

$$\begin{aligned} f_{ik}^1 &= f_{ik}^0 \sum_{jl} f_{jl}^0 [1 + (i/\sigma^2)d_{ik,jl}^0] \\ &= f_{ik}^0 + (i/\sigma^2) f_{ik}^0 [\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}]. \end{aligned}$$

The frequency of the progeny genotype  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  is

$$\begin{aligned} f_{ikjl}^1 f_{jl}^1 &= \{f_{ik}^0 + (i/\sigma^2)f_{ik}^0[\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}]\}\{f_{jl}^0 + (i/\sigma^2)f_{jl}^0[\alpha_j^1 + \alpha_l^2 + (\alpha\alpha)_{jl}]\} \\ &\cong f_{ik}^0 f_{jl}^0 + (i/\sigma^2)f_{ik}^0 f_{jl}^0[\alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2 + (\alpha\alpha)_{ik} + (\alpha\alpha)_{jl}]. \end{aligned}$$

The genotypic mean of the progeny population, then, is

$$\begin{aligned} \mu_1 &= \sum_{ijkl} f_{ik}^1 f_{jl}^1 d_{ik,jl}^0 \\ &\cong (i/\sigma^2)[\sigma_A^2 + \frac{1}{2}\sigma_{AA}^2]. \end{aligned}$$

It is immediately apparent that this mean value for  $\Pi_1$  is not the same as that predicted with the gene analysis. Thus, when it is considered that selection operates on genotypes at each locus separately, one cycle of selection results in an increment change of  $(i/\sigma^2)\sigma_{A(1)}^2$  for the first locus and  $(i/\sigma^2)\sigma_{A(2)}^2$  for the second locus. The total increment change due to selection operating separately on the two loci is, then,

$$\begin{aligned} \mu_1 &= (i/\sigma^2)[\sigma_{A(1)}^2 + \sigma_{A(2)}^2] \\ &= (i/\sigma^2)\sigma_A^2. \end{aligned}$$

Although it is clear that the more accurate prediction of the *immediate* results of one cycle of selection is that obtained by using the gamete rather than the gene analysis, the question remains as to what happens to the predicted mean value when selection is relaxed and mating is continued at random.

Briefly, the answer is as follows; if epistasis occurs, the progeny population,  $\Pi_1$ , is not in gene equilibrium, i.e.

$$\sum_{ijkl} f_{ik}^1 f_{jl}^1 (A_i^1 A_k^2)(A_j^1 A_l^2) \neq [\sum_{ij} (p_i^1)^1 (p_j^1)^1 A_i^1 A_j^1] \cdot [\sum_{kl} (p_k^2)^1 (p_l^2)^1 A_k^2 A_l^2],$$

or more simply

$$f_{uv}^1 \neq (p_u^1)^1 (p_v^2)^1,$$

where  $(p_u^1)^1$  is the frequency of  $A_u^1$  in  $\Pi_1$ .

Since the progeny population is not in gene equilibrium and since the gamete is a highly mutable unit of inheritance (i.e.  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  produces not only parental but also a relatively high frequency of non-parental gametes) continued random mating in the absence of selection causes the population structure to continually change until equilibrium is reached. In the case of two linked loci the speed of this change depends on the magnitude of the recombination frequency. As the structure of the population changes, the mean of the population decays until finally, at equilibrium, the population mean equals that which is predicted on the basis of the gene as a unit of inheritance. Then, assuming that the gene is stable and that natural selection is not operating, the population mean will remain at this value. The following develops the argument more rigorously.

Starting with the progeny population which results from one cycle of selection, the objective is to show what happens to the population structure and the population mean value when random mating is continued without selection. Again,



consider the parameters associated with the gene model as those defined for the population  $\Pi_0$  and, therefore, the superscript "0" will not be used. However, the gamete frequencies will have a more complicated superscript notation, i.e. let  $f_{ik}^{1,r}$  denote the frequency of the gamete ( $A_i^1 A_k^2$ ) in the  $r$ th generation of random mating without selection, after one cycle of selection.

The progeny population following one cycle of selection may be designated as

$$\Pi_{1,0} = \sum_{ijkl} f_{ik}^{1,0} f_{jl}^{1,0} (A_i^1 A_k^2) (A_j^1 A_l^2).$$

The total gametic array from  $\Pi_{1,0}$ , in the absence of selection, is

$$\begin{aligned} \sum_{ijkl} f_{ik}^{1,0} f_{jl}^{1,0} \{[(1-y)/2](A_i^1 A_k^2 + A_j^1 A_l^2) + (y/2)(A_i^1 A_l^2 + A_j^1 A_k^2)\} \\ = \sum_{ijkl} [(1-y)f_{ik}^{1,0} f_{jl}^{1,0} + y f_{il}^{1,0} f_{jk}^{1,0}] (A_i^1 A_k^2) \\ = \sum_{ik} f_{ik}^{1,1} (A_i^1 A_k^2), \end{aligned}$$

where

$$\begin{aligned} f_{ik}^{1,1} &= \sum_{jl} \{(1-y)f_{ik}^{1,0} f_{jl}^{1,0} + y f_{il}^{1,0} f_{jk}^{1,0}\} \\ &= (1-y)f_{ik}^{1,0} + y \sum_{jl} f_{il}^{1,0} f_{jk}^{1,0} \dots \dots \dots (1) \\ &= (1-y)\{f_{ik}^{0,0} + (i/\sigma^2)f_{ik}^{0,0}[\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}]\} \\ &\quad + y \sum_{jl} \{f_{il}^{0,0} + (i/\sigma^2)f_{il}^{0,0}[\alpha_i^1 + \alpha_l^2 + (\alpha\alpha)_{il}]\} \{f_{jk}^{0,0} + (j/\sigma^2)f_{jk}^{0,0}[\alpha_j^1 + \alpha_k^2 + (\alpha\alpha)_{jk}]\} \\ &\cong f_{ik}^{0,0} + (i/\sigma^2)f_{ik}^{0,0}[\alpha_i^1 + \alpha_k^2 + (1-y)(\alpha\alpha)_{ik}]. \end{aligned}$$

The mean of the population  $\Pi_{1,1}$  is then

$$\begin{aligned} \mu_{1,1} &= \sum_{ijkl} f_{ik}^{1,1} f_{jl}^{1,1} d_{ik,jl}^0 \\ &= \sum_{ijkl} \{f_{ik}^{0,0} + (i/\sigma^2)f_{ik}^{0,0}[\alpha_i^1 + \alpha_k^2 + (1-y)(\alpha\alpha)_{ik}]\} \\ &\quad \times \{f_{jl}^{0,0} + (j/\sigma^2)f_{jl}^{0,0}[\alpha_j^1 + \alpha_l^2 + (1-y)(\alpha\alpha)_{jl}]\} d_{ik,jl}^0 \\ &\cong (i/\sigma^2)[\sigma_A^2 + (1-y)\frac{1}{2}\sigma_{AA}^2]. \end{aligned}$$

An argument used by Kempthorne (1957) may be employed to predict the mean after an arbitrary number  $m$  of generations of random mating without selection.

Equation (1) may be rewritten as

$$f_{ik}^{1,1} = (1-y)f_{ik}^{1,0} + y \sum_l f_{il}^{1,0} \sum_j f_{jk}^{1,0},$$

where

$$\sum_l f_{il}^{1,0} = f_i^{1,0} = \text{frequency of } A_i^1 \text{ in } \Pi_{1,0},$$

and

$$\sum_j f_{jk}^{1,0} = f_k^{1,0} = \text{frequency of } A_k^2 \text{ in } \Pi_{1,0}.$$

In the absence of selection gene frequencies do not change, i.e.  $f_i^{1,1} = f_i^{1,0}$  etc. Therefore, the quantity  $(f_i^{1,1} \cdot f_{.k}^{1,1})$  may be subtracted from the right-hand side of (1) and  $(f_i^{1,0} \cdot f_{.k}^{1,0})$  may be subtracted from the left-hand side of the same equation. Thus,

$$\begin{aligned} f_{ik}^{1,1} - f_i^{1,1} f_{.k}^{1,1} &= (1-y) f_{ik}^{1,0} + y f_i^{1,0} f_{.k}^{1,0} - f_i^{1,0} f_{.k}^{1,0} \\ &= (1-y) (f_{ik}^{1,0} - f_i^{1,0} f_{.k}^{1,0}), \end{aligned}$$

or

$$\Delta_{ik}^{1,1} = (1-y) \Delta_{ik}^{1,0}.$$

Likewise

$$\Delta_{ik}^{1,2} = (1-y) \Delta_{ik}^{1,1}.$$

$$= (1-y)^2 \Delta_{ik}^{1,0},$$

and more generally

$$\Delta_{ik}^{1,m} = (1-y)^m \Delta_{ik}^{1,0},$$

or

$$f_{ik}^{1,m} - f_i^{1,m} f_{.k}^{1,m} = (1-y)^m (f_{ik}^{1,0} - f_i^{1,0} f_{.k}^{1,0}).$$

Since  $f_i^{1,m} = f_i^{1,0}$ , etc.

$$f_{ik}^{1,m} = f_i^{1,0} f_{.k}^{1,0} + (1-y)^m (f_{ik}^{1,0} - f_i^{1,0} f_{.k}^{1,0}). \quad \dots\dots\dots (2)$$

Recalling that

$$f_i^{1,0} = p_i^1 [1 + (i/\sigma^2) \alpha_i^1],$$

$$f_{.k}^{1,0} = p_k^2 [1 + (i/\sigma^2) \alpha_k^2],$$

and

$$f_{ik}^{1,0} = f_{ik}^{0,0} \{1 + (i/\sigma^2) [\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}]\},$$

equation (2) becomes,

$$f_{ik}^{1,m} \cong f_{ik}^{0,0} \{1 + (i/\sigma^2) [\alpha_i^1 + \alpha_k^2 + (1-y)^m (\alpha\alpha)_{ik}]\}.$$

The mean of the population which is submitted to one cycle of selection and then to  $m$  generations of random mating without selection is

$$\begin{aligned} \mu_{1,m} &= \sum_{ijk} f_{ik}^{1,m} f_{jl}^{1,m} d_{ik,jl}^0 \\ &\cong (i/\sigma^2) [\sigma_A^2 + (1-y)^m \frac{1}{2} \sigma_{AA}^2]. \end{aligned}$$

Thus, with random mating without selection, the mean of the population  $\Pi_{1,0}$  decays toward the lower asymptote which is

$$\lim_{m \rightarrow \infty} (\mu_{1,m}) \rightarrow (i/\sigma^2) \sigma_A^2.$$

This is the value predicted by the gene analysis.

The change in population mean may also be described by the use of the increment mean values. Thus, if the increment change for means in the  $(m-1)$ th

and  $m$ th populations is defined as

$${}_1\Delta_{(m-1),m} = \mu_{1,(m-1)} - \mu_{1,m},$$

then

$$\begin{aligned} {}_1\Delta_{(m-1),m} &= (i/\sigma^2)\{(1-y)^{m-1}[1-(1-y)]\} \frac{1}{2}\sigma_{AA}^2 \\ &= (i/\sigma^2)[y(1-y)^{m-1}] \frac{1}{2}\sigma_{AA}^2. \end{aligned}$$

Hence

$$\lim_{m \rightarrow \infty} [{}_1\Delta_{(m-1),m}] \rightarrow 0.$$

(iii) *Consequences of Two or More Cycles of Continuous Selection.*—The objective in this section is to describe the changes in parameters which occur with an arbitrary number of continuous cycles of selection. The procedure will be to outline briefly the normal method of obtaining  $\Pi_2$  by selecting in  $\Pi_1$ . Then the problem of generalizing to  $n$  generations of continuous selection will be attacked.

In the previous section it was shown that one generation of selection yielded the following population

$$\Pi_1 = \sum_{ijkl} f_{ik}^1 f_{jl}^1 (A_i^1 A_k^2) (A_j^1 A_l^2).$$

After selection, the frequency of  $(A_i^1 A_k^2) (A_j^1 A_l^2)$  is

$$\begin{aligned} f_{ik}^1 f_{jl}^1 w_{ik,jl}^1 &\cong f_{ik}^1 f_{jl}^1 w_{ik,jl}^0 \\ &= f_{ik}^1 f_{jl}^1 [1 + (i/\sigma^2) d_{ik,jl}^0]. \end{aligned}$$

The total array of gametes from the selected genotypes is

$$\begin{aligned} \sum_{ijkl} f_{ik}^1 f_{jl}^1 w_{ik,jl}^0 \{[(1-y)/2](A_i^1 A_k^2 + A_j^1 A_l^2) + (y/2)(A_i^1 A_l^2 + A_j^1 A_k^2)\} \\ = \sum_{ik} f_{ik}^2 (A_i^1 A_k^2), \end{aligned}$$

where

$$\begin{aligned} f_{ik}^2 &= \sum_{jl} [(1-y) f_{ik}^1 f_{jl}^1 w_{ik,jl}^0 + y f_{il}^1 f_{jk}^1 w_{il,jk}^0] \\ &= A + B. \end{aligned}$$

The  $A$  term may be evaluated as follows:

$$\begin{aligned} A &= (1-y) \sum_{jl} \{f_{ik}^0 f_{jl}^0 + (i/\sigma^2) f_{ik}^0 f_{jl}^0 [\alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2 + (\alpha\alpha)_{ik} + (\alpha\alpha)_{jl}] + (i/\sigma^2) f_{ik}^0 f_{jl}^0 d_{ik,jl}^0\} \\ &= (1-y) \{f_{ik}^0 + 2(i/\sigma^2) f_{ik}^0 [\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}]\}. \end{aligned}$$

The  $B$  term yields

$$\begin{aligned} B &= y \sum_{jl} \{f_{il}^0 f_{jk}^0 + (i/\sigma^2) f_{il}^0 f_{jk}^0 [\alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2 + (\alpha\alpha)_{il} + (\alpha\alpha)_{jk}] + (i/\sigma^2) f_{il}^0 f_{jk}^0 d_{il,jk}^0\} \\ &= y \{f_{ik}^0 + (i/\sigma^2) f_{ik}^0 (\alpha_i^1 + \alpha_k^2) + (i/\sigma^2) f_{ik}^0 [\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}]\}. \end{aligned}$$

Hence

$$f_{ik}^2 = f_{ik}^0 + 2(i/\sigma^2)f_{ik}^0[\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] - y(i/\sigma^2)f_{ik}^0(\alpha\alpha)_{ik}.$$

The mean of  $\Pi_2$  is, then,

$$\begin{aligned}\mu_2 &= \sum_{ijkl} f_{ik}^2 f_{jl}^2 d_{ik,jl}^0 \\ &\cong \sum_{ijkl} \{f_{ik}^0 f_{jl}^0 + 2(i/\sigma^2)f_{ik}^0 f_{jl}^0[\alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2 + (\alpha\alpha)_{ik} + (\alpha\alpha)_{jl}] \\ &\quad - y(i/\sigma^2)f_{ik}^0 f_{jl}^0[(\alpha\alpha)_{ik} + (\alpha\alpha)_{jl}]\} d_{ik,jl}^0 \\ &= 2(i/\sigma^2)(\sigma_A^2 + \frac{1}{2}\sigma_{AA}^2) - y(i/\sigma^2)\frac{1}{2}\sigma_{AA}^2 \\ &= (i/\sigma^2)[\sigma_A^2 + \frac{1}{2}\sigma_{AA}^2] + (i/\sigma^2)[\sigma_A^2 + (1-y)\frac{1}{2}\sigma_{AA}^2].\end{aligned}$$

The final problem is to generalize the results to  $n$  generations of continuous selection. Thus, it is required to give the approximate value for the mean of  $\Pi_n$  in terms of the parameters of  $\Pi_0$ . To do this it is necessary to express the frequency of the gamete  $(A_i^1 A_k^2)$  in the  $n$ th generation in terms of the frequency of the same gamete in  $\Pi_0$ .

The first step is to obtain a recurrence relation involving  $f_{ik}^n$  and  $f_{ik}^{n-1}$ . Consider, then, the  $(n-1)$ th generation, i.e.

$$\Pi_{(n-1)} = \sum_{ijkl} f_{ik}^{n-1} f_{jl}^{n-1} (A_i^1 A_k^2)(A_j^1 A_l^2).$$

Following selection, the frequency of  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  is

$$f_{ik}^{n-1} f_{jl}^{n-1} w_{ik,jl}^{n-1} \cong f_{ik}^{n-1} f_{jl}^{n-1} w_{ik,jl}^0.$$

The total array of gametes from the selected parents is

$$\begin{aligned}\sum_{ijkl} f_{ik}^{n-1} f_{jl}^{n-1} w_{ik,jl}^0 &\{[(1-y)/2](A_i^1 A_k^2 + A_j^1 A_l^2) + (y/2)(A_i^1 A_l^2 + A_j^1 A_k^2)\} \\ &= \sum_{ik} f_{ik}^n (A_i^1 A_k^2),\end{aligned}$$

where

$$\begin{aligned}f_{ik}^n &= \sum_{jl} [(1-y)f_{ik}^{n-1} f_{jl}^{n-1} w_{ik,jl}^0 + y f_{il}^{n-1} f_{jk}^{n-1} w_{il,jk}^0] \\ &= A + B.\end{aligned}$$

The terms  $A$  and  $B$  may be evaluated as follows:

$$A = (1-y)f_{ik}^{n-1} + (1-y)(i/\sigma^2)f_{ik}^{n-1} \sum_{jl} f_{jl}^{n-1} d_{ik,jl}^0,$$

and

$$B = y f_{il}^{n-1} f_{jk}^{n-1} + y(i/\sigma^2) \sum_{jl} f_{il}^{n-1} f_{jk}^{n-1} d_{il,jk}^0.$$

Hence

$$f_{ik}^n = (1-y)f_{ik}^{n-1} + y f_{il}^{n-1} f_{jk}^{n-1} + (1-y)(i/\sigma^2)f_{ik}^{n-1} \sum_{jl} f_{jl}^{n-1} d_{ik,jl}^0 + y(i/\sigma^2) \sum_{jl} f_{il}^{n-1} f_{jk}^{n-1} d_{il,jk}^0.$$



In order to evaluate  $f_{ik}^n$  in terms of the parameters of  $\Pi_0$ , consider, first, the frequencies of  $f_{ik}$  for the first few cycles:

(i)  $n = 1$

$$f_{ik}^1 = (1-y)f_{ik}^0 + (i/\sigma^2)f_{ik}^0[\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] + yf_{ik}^0.$$

(ii)  $n = 2$

$$f_{ik}^2 = (1-y)^2f_{ik}^0 + [1 + (1-y)](i/\sigma^2)f_{ik}^0[\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] \\ + [1 + (1-y)]yf_{ik}^0 + y(i/\sigma^2)f_{ik}^0(\alpha_i^1 + \alpha_k^2).$$

(iii)  $n = 3$

$$f_{ik}^3 = (1-y)^3f_{ik}^0 + [1 + (1-y) + (1-y)^2](i/\sigma^2)f_{ik}^0[\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] \\ + [1 + (1-y) + (1-y)^2]yf_{ik}^0 + [2 + (1-y)]y(i/\sigma^2)f_{ik}^0(\alpha_i^1 + \alpha_k^2).$$

(iv)  $n = 4$

$$f_{ik}^4 = (1-y)^4f_{ik}^0 + [1 + (1-y) + (1-y)^2 + (1-y)^3](i/\sigma^2)f_{ik}^0[\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] \\ + [1 + (1-y) + (1-y)^2 + (1-y)^3]yf_{ik}^0 + [3 + 2(1-y) + (1-y)^2]y(i/\sigma^2)f_{ik}^0(\alpha_i^1 + \alpha_k^2).$$

It appears that, in general,

$$f_{ik}^n = (1-y)^nf_{ik}^0 + \left[ \sum_{r=1}^n (1-y)^{r-1} y f_{ik}^0 + \left[ \sum_{r=1}^n (1-y)^{r-1} (i/\sigma^2) f_{ik}^0 [\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] \right] \right. \\ \left. + \left\{ \sum_{t=1}^{n-1} \left[ \sum_{r=1}^t (1-y)^{r-1} y \right] (i/\sigma^2) f_{ik}^0 (\alpha_i^1 + \alpha_k^2) \right\} \right] \\ = \{ (1-y)^n + y[1 - (1-y)^n]/y \} f_{ik}^0 + [(1 - (1-y)^n)/y] (i/\sigma^2) f_{ik}^0 [\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] \\ + \left\{ \sum_{t=1}^{n-1} [(1 - (1-y)^t)/y] y \right\} (i/\sigma^2) f_{ik}^0 (\alpha_i^1 + \alpha_k^2) \\ = f_{ik}^0 + [(1 - (1-y)^n)/y] (i/\sigma^2) f_{ik}^0 [\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] + \{ n - [(1 - (1-y)^n)/y] \} (i/\sigma^2) f_{ik}^0 (\alpha_i^1 + \alpha_k^2) \\ = f_{ik}^0 + n(i/\sigma^2) f_{ik}^0 (\alpha_i^1 + \alpha_k^2) + [(1 - (1-y)^n)/y] (i/\sigma^2) f_{ik}^0 [(\alpha\alpha)_{ik}].$$

Having obtained an expression relating the frequency of the gamete ( $A_i^1 A_k^2$ ) in the  $n$ th generation to parameters in  $\Pi_0$ , it is possible to evaluate the mean of  $\Pi_n$  in terms of the parameters of  $\Pi_0$ . Thus,

$$\mu_n = \sum_{ijkl} f_{ik}^n f_{jl}^n d_{ik,jl}^0 \\ = \sum_{ijkl} \{ f_{ik}^0 + n(i/\sigma^2) f_{ik}^0 (\alpha_i^1 + \alpha_k^2) + [(1 - (1-y)^n)/y] (i/\sigma^2) f_{ik}^0 [(\alpha\alpha)_{ik}] \} \\ \times \{ f_{jl}^0 + n(i/\sigma^2) f_{jl}^0 (\alpha_j^1 + \alpha_l^2) + [(1 - (1-y)^n)/y] (i/\sigma^2) f_{jl}^0 [(\alpha\alpha)_{jl}] \} \times d_{ik,jl}^0 \\ \cong \sum_{ijkl} \{ f_{ik}^0 f_{jl}^0 + n(i/\sigma^2) f_{ik}^0 f_{jl}^0 (\alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2) + [1 - (1-y)^n]/y \} \\ \times (i/\sigma^2) f_{ik}^0 f_{jl}^0 [(\alpha\alpha)_{ik} + (\alpha\alpha)_{jl}] \times d_{ik,jl}^0 \\ = n(i/\sigma^2) (\sigma_A^2) + [(1 - (1-y)^n)/y] (i/\sigma^2) \frac{1}{2} \sigma_{AA}^2,$$

or

$$\begin{aligned}\mu_n &= (i/\sigma^2)[n\sigma_A^2 + \sum_{r=1}^n (1-y)^{r-1} \frac{1}{2}\sigma_{AA}^2] \\ &= (i/\sigma^2)[\sigma_A^2 + \frac{1}{2}\sigma_{AA}^2] + (i/\sigma^2)[\sigma_A^2 + (1-y)\frac{1}{2}\sigma_{AA}^2] + \dots \\ &\quad + \dots + (i/\sigma^2)[\sigma_A^2 + (1-y)^{n-1}\frac{1}{2}\sigma_{AA}^2].\end{aligned}$$

It is clear that the extent of the influence of  $\sigma_{AA}^2$  is largely determined by the magnitude of the recombination value which has the range  $0 \leq y \leq \frac{1}{2}$ . Thus if  $y=0$  (i.e. no recombination),

$$\mu_n = (i/\sigma^2)[n\sigma_A^2 + n(\frac{1}{2}\sigma_{AA}^2)],$$

and if  $y = \frac{1}{2}$  (loci independent),

$$\mu_n = (i/\sigma^2)\{n\sigma_A^2 + [2 - (\frac{1}{2})^{n-1}]\frac{1}{2}\sigma_{AA}^2\}.$$

Therefore, if the loci exhibit a low recombination value, considerable effect can be generated by  $\sigma_{AA}^2$ , and if the loci are independent the maximum contribution after  $n$  generations of selection is approximately  $\sigma_{AA}^2$  (with large  $n$ ).

It is also clear that even with constant selection differential and quite apart from the disturbance due to approximations, the increment changes between consecutive cycles of selection are not equal (assuming  $y \neq 0$ ). Thus, if  $\Delta\mu_{n,n-1} = \mu_n - \mu_{n-1}$ , then

$$\Delta\mu_{1,0} = (i/\sigma^2)[\sigma_A^2 + \frac{1}{2}\sigma_{AA}^2],$$

$$\Delta\mu_{2,1} = (i/\sigma^2)[\sigma_A^2 + (1-y)\frac{1}{2}\sigma_{AA}^2],$$

and more generally

$$\Delta\mu_{n,n-1} = (i/\sigma^2)[\sigma_A^2 + (1-y)^{n-1}\frac{1}{2}\sigma_{AA}^2].$$

Hence, the influence of  $\sigma_{AA}^2$  diminishes as the number of cycles in the selection programme increases. This causes a departure from linearity of the response of selection with time.

(iv) *Consequences of Relaxation of Selection after n Cycles of Continuous Selection.*—The objective in this last part of the section dealing with the consequences of selection operating on genotypes generated by alleles at two loci is to develop the prediction equation for the mean of a population which is submitted to  $n$  generations of continuous selection and then to  $m$  generations of random mating without selection.

In the last section it was shown that the population after  $n$  continuous cycles of selection had the following structure

$$\Pi_{n,0} = \sum_{ijkl} f_{ik}^{n,0} f_{jl}^{n,0} (A_i^1 A_k^2) (A_j^1 A_l^2).$$

If, now, mating is at random, in the absence of selection, the frequency of the gamete ( $A_i^1 A_k^2$ ) in the next generation is

$$f_{ik}^{n,1} = (1-y)f_{ik}^{n,0} + yf_{i.}^{n,0}f_{.k}^{n,0}.$$

Since gene frequencies do not change, this equation may be rewritten as

$$f_{ik}^{n,1} - f_{i.}^{n,1}f_{.k}^{n,1} = (1-y)(f_{ik}^{n,0} - f_{i.}^{n,0}f_{.k}^{n,0}),$$

which may be symbolized as

$$\Delta_{ik}^{n,1} = (1-y)\Delta_{ik}^{n,0}.$$

More generally

$$\Delta_{ik}^{n,m} = (1-y)^m \Delta_{ik}^{n,0},$$

or

$$f_{ik}^{n,m} = f_{i.}^{n,0}f_{.k}^{n,0} + (1-y)^m(f_{ik}^{n,0} - f_{i.}^{n,0}f_{.k}^{n,0}). \quad (3)$$

Recalling that

$$f_{i.}^{n,0} = p_i^1 + n(i/\sigma^2)p_i^1\alpha_i^1,$$

$$f_{.k}^{n,0} = p_k^2 + n(i/\sigma^2)p_k^2\alpha_k^2,$$

and

$$f_{ik}^{n,0} = f_{jk}^{0,0} + a(i/\sigma^2)f_{ik}^{0,0}[\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] + b(i/\sigma^2)f_{ik}^{0,0}(\alpha_i^1 + \alpha_k^2),$$

where

$$a = [(1 - (1-y)^n)/y],$$

and

$$b = \{n - [(1 - (1-y)^n)/y]\},$$

equation (3) becomes

$$\begin{aligned} f_{ik}^{n,m} &= f_{ik}^{0,0} + n(i/\sigma^2)f_{ik}^{0,0}(\alpha_i^1 + \alpha_k^2) + (1-y)^m\{a(i/\sigma^2)f_{ik}^{0,0}[\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] \\ &\quad + (b-n)(i/\sigma^2)f_{ik}^{0,0}(\alpha_i^1 + \alpha_k^2)\}. \end{aligned}$$

It is now possible to obtain the mean of the population that has had a history of  $n$  generations of selection followed by  $m$  generations of random mating without selection. This mean is

$$\begin{aligned} \mu_{n,m} &= \sum_{ijkl} f_{ik}^{n,m} f_{jl}^{n,m} d_{ik,jl}^0 \\ &\cong \sum_{ijkl} \{f_{ik}^{0,0}f_{jl}^{0,0} + n(i/\sigma^2)f_{ik}^{0,0}f_{jl}^{0,0}(\alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2) + (1-y)^m a(i/\sigma^2) \\ &\quad \times f_{ik}^{0,0}f_{jl}^{0,0}[\alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2 + (\alpha\alpha)_{ik} + (\alpha\alpha)_{jl}] \\ &\quad + (1-y)^m(b-n)(i/\sigma^2)f_{ik}^{0,0}f_{jl}^{0,0}(\alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2)\} \times d_{ik,jl}^0 \\ &= n(i/\sigma^2)\sigma_A^2 + (1-y)^m a(i/\sigma^2)(\sigma_A^2 + \frac{1}{2}\sigma_{AA}^2) + (1-y)^m(b-n)(i/\sigma^2)\sigma_A^2 \\ &= n(i/\sigma^2)\sigma_A^2 + [(1 - (1-y)^n)/y](1-y)^m(i/\sigma^2)\frac{1}{2}\sigma_{AA}^2, \end{aligned}$$

or

$$\begin{aligned} \mu_{n,m} &= n(i/\sigma^2)\sigma_A^2 + (1-y)^m \left[ \sum_{r=1}^n (1-y)^{r-1} (i/\sigma^2)\frac{1}{2}\sigma_{AA}^2 \right. \\ &\quad \left. + (i/\sigma^2)[\sigma_A^2 + (1-y)^m \frac{1}{2}\sigma_{AA}^2] + (i/\sigma^2)[\sigma_A^2 + (1-y)^m(1-y)\frac{1}{2}\sigma_{AA}^2] \right. \\ &\quad \left. + \dots + (i/\sigma^2)[\sigma_A^2 + (1-y)^m(1-y)^{n-1}\frac{1}{2}\sigma_{AA}^2] \right]. \end{aligned}$$

If the increment change in means in the  $(m-1)$ th and  $m$ th populations is defined as

$$n\Delta\mu_{(m-1),m} = \mu_{n,m-1} - \mu_{n,m},$$

then

$$\begin{aligned} n\Delta\mu_{(m-1),m} &= \{[(1-(1-y)^n)/y](1-y)^{m-1} - [(1-(1-y)^n)/y](1-y)^m\} (i/\sigma^2) \frac{1}{2}\sigma_{AA}^2 \\ &= [(1-(1-y)^n)/y](1-y)^{m-1} [1-(1-y)] (i/\sigma^2) \frac{1}{2}\sigma_{AA}^2 \\ &= (1-y)^{m-1} [1-(1-y)^n] (i/\sigma^2) \frac{1}{2}\sigma_{AA}^2. \end{aligned}$$

When  $n = 1$ ,

$$1\Delta\mu_{(m-1),m} = (1-y)^{m-1} y (i/\sigma^2) \frac{1}{2}\sigma_{AA}^2,$$

as found in a previous section.

It is quite clear that for any value of  $n$ ,

$$\lim_{m \rightarrow \infty} (n\Delta\mu_{(m-1),m}) \rightarrow 0.$$

Thus, the mean of the population  $\Pi_{n,0}$  decays to

$$\lim_{m \rightarrow \infty} (\mu_{n,m}) \rightarrow n(i/\sigma^2) \sigma_{AA}^2.$$

Again it must be pointed out that in this analysis it is assumed that natural selection is not operating.

### (c) Generalizations

The approach which utilizes a gamete analysis with a gene interpretation, as set out for the two-locus case, can be extended to include more loci. However, the problem becomes immensely difficult due, primarily, to the increase in the number of linkage parameters. For example, with three loci there are three recombination values which must be considered. It is for this reason that a somewhat different approach is used.

By introducing the notion of the individual as a unit of inheritance and utilizing certain elements of the gamete analysis, complete generalization can be obtained for at least some descriptive aspects of the response to truncation selection. Clearly, however, a generalized analysis based on individuals and gametes as units of inheritance cannot yield a detailed description such as that developed in the previous sections, unless, of course, a gene interpretation is made. This requires that the genetic structure is specified in terms of genes, gene loci, and linkage parameters.

In the following, the first step is to briefly set out the generalized analysis. Then an interpretation of this analysis is made in terms of the gene system which involves two linked loci, the system which has been treated in detail in Section II(b). This gene interpretation is made for two reasons, (i) to illustrate the versatility of methods which may be employed by utilizing different levels of genetic organization as units of inheritance, and (ii) to show that the generalized analysis does give detailed results if the gene structure is specified.



An attack on the generalized description of the change in the population mean, due to selection, is made by using the individual as the basic unit of inheritance and specifying the frequency of the individual in terms of the frequencies of the gametes which united to form the individual. The argument starts with definitions which apply to the elements in the original random-mating population in equilibrium,  $\Pi_0$ .

Let

$$\sum f_i G_i = \text{gametic array,}$$

$$H_{ij} = \text{genotype which results from the union of } G_i \text{ and } G_j,$$

$$\sum_{ij} f_i^0 f_j^0 H_{ij} = \text{genotypic array,}$$

and

$$h_{ij} = \text{genotypic value of } H_{ij}, \text{ such that } \sum_{ij} f_i^0 f_j^0 h_{ij} = 0.$$

To define the mean of a full-sib array, further definitions are required.

Let

$$\sum_k p_{ijk} G_{ijk} = \text{gametic array produced by } H_{ij},$$

$$\sum_n p_{lmn} G_{lmn} = \text{gametic array produced by } H_{lm},$$

$$H_{ijk.lmn} = \text{genotype resulting from the union of gametes } G_{ijk} \text{ and } G_{lmn},$$

and

$$h_{ijk.lmn} = \text{genotypic value of } H_{ijk.lmn}, \text{ such that}$$

$$\sum_{ijklmn} f_i^0 f_j^0 f_k^0 f_l^0 f_m^0 f_n^0 p_{ijk} p_{lmn} h_{ijk.lmn} = 0.$$

The full-sib array which results from the cross between an arbitrary sire,  $H_{ij}$ , and an arbitrary dam,  $H_{lm}$ , is, then,

$$H_{(ij).(lm)} = \sum_{kn} p_{ijk} p_{lmn} H_{ijk.lmn},$$

and the mean of this array is

$$\bar{h}_{(ij).(lm)} = \sum_{kn} p_{ijk} p_{lmn} h_{ijk.lmn}.$$

Thus, the random mating-population in equilibrium,  $\Pi_0$ , can be generated entirely in terms of the individual as a unit of inheritance as follows:

$$\Pi_0 = \sum_{ijlm} (f_i^0 f_j^0)(f_l^0 f_m^0) H_{(ij).(lm)}.$$

The mean of  $\Pi_0$  is then

$$\mu_0 = \sum_{ijlm} (f_i^0 f_j^0)(f_l^0 f_m^0) \bar{h}_{(ij).(lm)} = 0.$$

The consequences of selection may now be considered. First, denote the frequency of  $H_{ij}$  in the selected parents from  $\Pi_0$  as

$$\begin{aligned} P_{ij}^1 &= (f_i^0 f_j^0) w_{ij}^0 \\ &= f_i^0 f_j^0 + (i/\sigma^2) f_i^0 f_j^0 h_{ij}^0. \end{aligned}$$

The mean of the selected parents is then,

$$\begin{aligned}\mu_s &= \sum_{ij} P_{ij}^1 h_{ij}^0 \\ &= \sum_{ij} [f_i^0 f_j^0 + (i/\sigma^2) f_i^0 f_j^0 h_{ij}^0] h_{ij}^0 \\ &= i(\sigma_G^2/\sigma^2).\end{aligned}$$

The mean of the progeny population,  $\Pi_1$ , may be determined as follows:

$$\begin{aligned}\mu_{1,0} &= \sum_{ijlm} P_{ij}^1 P_{lm}^1 h_{(ij)(lm)}^0 \\ &\cong \sum_{ijlm} \{(f_i^0 f_j^0)(f_l^0 f_m^0) + (i/\sigma^2)(f_i^0 f_j^0)(f_l^0 f_m^0)(h_{ij}^0 + h_{lm}^0)\} h_{(ij)(lm)}^0 \\ &= \sum_{ijlm} (f_i^0 f_j^0)(f_l^0 f_m^0) h_{(ij)(lm)}^0 + (i/\sigma^2) \sum_{ijlm} (f_i^0 f_j^0)(f_l^0 f_m^0)(h_{ij}^0 + h_{lm}^0) h_{(ij)(lm)}^0 \\ &= i\{[2 \text{Cov(PO)}]/\sigma^2\}.\end{aligned}$$

If epistasis occurs, this predicted mean value is not the same as that predicted on the basis of a gene analysis, i.e.

$$\mu_1 = i(\sigma_A^2/\sigma^2).$$

However, if random mating is imposed without selection, then the mean,  $i\{[2 \text{Cov(PO)}]/\sigma^2\}$ , regresses to

$$\lim_{m \rightarrow \infty} (\mu_{1,m}) \rightarrow i(\sigma_A^2/\sigma^2).$$

The means,  $\mu_{1,0}$  and  $\lim_{m \rightarrow \infty} (\mu_{1,m})$ , may be predicted as follows:

$$\mu_{1,0} = i\{[2 \text{Cov(PO)}]/\sigma^2\},$$

and

$$\lim_{m \rightarrow \infty} (\mu_{1,m}) = i(\sigma_A^2/\sigma^2) = i \text{ (heritability in the narrow sense).}$$

Consider, now, a second cycle of selection. The population  $\Pi_1$  may be generated as

$$\sum_{ijlm} P_{ij}^1 P_{lm}^1 H_{(ij)(lm)}.$$

However, this representation, involving only individual genotypes and their frequencies, does not specify the frequency of a given genotype,  $H_{ij}$ , in  $\Pi_1$ . This frequency must be specified by the frequency of gametes which unite to form  $H_{ij}$ , i.e.  $(f_i^1 f_j^1)$ . Hence, if  $P_{ij}^2$  denotes the frequency of  $H_{ij}$  in the population selected from  $\Pi_1$ , then

$$\begin{aligned}P_{ij}^2 &= (f_i^1 f_j^1) w_{ij}^1 \\ &\cong f_i^1 f_j^1 [1 + (i/\sigma^2) h_{ij}^0].\end{aligned}$$

The mean of the progeny population,  $\Pi_2$ , is then

$$\begin{aligned}\mu_2 &= \sum_{ijlm} P_{ij}^2 P_{lm}^2 h_{(ij.) (lm.)}^0 \\ &\cong \sum_{ijlm} (f_i^1 f_j^1) (f_l^1 f_m^1) [1 + (i/\sigma^2)(h_{ij}^0 + h_{lm}^0)] h_{(ij.) (lm.)}^0 \\ &= \sum_{ijlm} (f_i^1 f_j^1) (f_l^1 f_m^1) h_{(ij.) (lm.)}^0 + (i/\sigma^2) \sum_{ijlm} (f_i^1 f_j^1) (f_l^1 f_m^1) (h_{ij}^0 + h_{lm}^0) h_{(ij.) (lm.)}^0 \\ &= A + B.\end{aligned}$$

The  $A$  term is, by definition, the mean of a population which is subjected to one cycle of selection and then to one generation of random mating without selection. Thus

$$A = \mu_{1,1}.$$

Since

$$f_i^1 = f_i^0 + (i/\sigma^2) K_i,$$

where  $K_i$  is a constant, the  $B$  term is approximately equal to

$$\begin{aligned}&(i/\sigma^2) \sum_{ijlm} (f_i^0 f_j^0) (f_l^0 f_m^0) (h_{ij}^0 + h_{lm}^0) h_{(ij.) (lm.)}^0 \\ &= i\{[2 \text{Cov(PO)}]/\sigma^2\}.\end{aligned}$$

Thus

$$\mu_{2,0} = \mu_{1,1} + i\{[2 \text{Cov(PO)}]/\sigma^2\}.$$

The mean of the population which has undergone  $n$  cycles of selection may be obtained by an extension of the above argument. If  $P_{ij}^n$  is the frequency of  $H_{ij}$  in the selected population from  $\Pi_{n-1}$ , then the mean of  $\Pi_n$  is

$$\mu_{n,0} = \sum_{ijlm} P_{ij}^n P_{lm}^n h_{(ij.) (lm.)}^0,$$

where

$$P_{ij}^n \cong (f_i^{n-1} f_j^{n-1}) w_{ij}^0.$$

Hence

$$\begin{aligned}\mu_n &\cong \sum_{ijlm} [(f_i^{n-1} f_j^{n-1}) w_{ij}^0] [(f_l^{n-1} f_m^{n-1}) w_{lm}^0] h_{(ij.) (lm.)}^0 \\ &= \sum_{ijlm} (f_i^{n-1} f_j^{n-1}) (f_l^{n-1} f_m^{n-1}) [1 + (i/\sigma^2)(h_{ij}^0 + h_{lm}^0)] h_{(ij.) (lm.)}^0 \\ &= \mu_{(n-1),1} + i\{[2 \text{Cov(PO)}]/\sigma^2\}.\end{aligned}$$

The increment difference between the means for the  $(n-1)$ th and the  $n$ th generation is

$$\begin{aligned}\Delta\mu_{n,n-1} &= \mu_n - \mu_{n-1} \\ &= \mu_{(n-1),1} - \mu_{(n-2),1}.\end{aligned}$$

Generally speaking, then, the mean of the progeny population which results from random mating selected parents from the previous population is equal to

the summation of two parts: (i) the mean of the previous population after it has been allowed to mate at random without selection for one generation, plus (ii) the increment  $i\{2 \text{Cov}(PO)/\sigma^2\}$ . If estimation is desired, both of these quantities are easily estimated and do not require a gene interpretation.

The consequences of relaxation of selection cannot be carried very far in a generalized formulation since it is not possible to obtain a recurrence relation for gamete frequencies in consecutive generations without specifying the gene constitution. However, the following general statements can be made.

The mean of the population that has had a history of  $n$  consecutive cycles of selection, followed by  $m$  generations of random mating without selection is

$$\mu_{n,m} = \sum_{ijklm} (f_i^{n,m-1} f_j^{n,m-1}) (f_l^{n,m-1} f_m^{n,m-1}) h_{(ij.)}^0 h_{(lm.)}^0.$$

If

$$n\Delta\mu_{(m-1),m} = \mu_{n,m-1} - \mu_{n,m},$$

then

$$\begin{aligned} n\Delta\mu_{(m-1),m} &= \sum_{ijklm} \{[(f_i^{n,m-2} f_j^{n,m-2})(f_l^{n,m-2} f_m^{n,m-2})] \\ &\quad - [f_i^{n,m-1} f_j^{n,m-1})(f_l^{n,m-1} f_m^{n,m-1})]\} h_{(ij.)}^0 h_{(lm.)}^0 \\ &= \sum \{n\Delta(\text{mating frequency})\} h_{(ij.)}^0 h_{(lm.)}^0. \end{aligned}$$

As  $m$  increases, the gamete frequency tends to the product of the component gene frequencies, and

$$n\Delta(\text{mating frequency}) \rightarrow 0,$$

and

$$n\Delta\mu_{(m-1),m} \rightarrow 0,$$

$$\mu_{n,m} \rightarrow i(\sigma_A^2/\sigma^2).$$

All of the results so far presented are valid for a completely general situation including any number of alleles at each of any number of loci which are associated in an arbitrary system of linkages. Also any system of dominance and epistatic values may be involved.

These generalized results will now be evaluated for the specific two-locus case.

For two loci, the mean of a population submitted to  $n$  cycles of selection was found to be

$$\mu_{n,0} = n(i/\sigma^2)\sigma_A^2 + [\sum_{r=1}^n (1-y)^{r-1}](i/\sigma^2)\frac{1}{2}\sigma_{AA}^2.$$

In generalized terms the same mean is represented as

$$\mu_{n,0} = (i/\sigma^2)[2 \text{Cov}(PO)] + \mu_{(n-1),1},$$

where, for two loci,

$$2 \text{Cov}(PO) = \sigma_A^2 + \frac{1}{2}\sigma_{AA}^2,$$

and

$$\mu_{(n-1),1} = (n-1)(i/\sigma^2)\sigma_A^2 + (1-y)[\sum_{r=1}^{n-1} (1-y)^{r-1}](i/\sigma^2)\frac{1}{2}\sigma_{AA}^2.$$



Hence

$$\mu_{n,0} = (i/\sigma^2)(\sigma_A^2 + \frac{1}{2}\sigma_{AA}^2) + (n-1)(i/\sigma^2)\sigma_A^2 + (1-y)\left[\sum_{r=1}^{n-1} (1-y)^{r-1}\right](i/\sigma^2)\frac{1}{2}\sigma_{AA}^2.$$

But this is clearly equal to

$$n(i/\sigma^2)\sigma_A^2 + \left[\sum_{r=1}^n (1-y)^{r-1}\right](i/\sigma^2)\frac{1}{2}\sigma_{AA}^2.$$

Thus, the generalized representation yields the required specific result for two loci.

Next, the general analysis yields the following equation for the increment change, due to selection, in the means of the  $(n-1)$ th and  $n$ th populations:

$$\Delta\mu_{n,n-1} = \mu_{(n-1),1} - \mu_{(n-2),1}.$$

Evaluation of this equation for the two-locus case may be made as follows:

$$\begin{aligned}\Delta\mu_{n,n-1} &= \{(n-1)(i/\sigma^2)\sigma_A^2 + (1-y)[(1-(1-y)^{n-1})/y](i/\sigma^2)\frac{1}{2}\sigma_{AA}^2\} \\ &\quad - \{(n-2)(i/\sigma^2)\sigma_A^2 + (1-y)[(1-(1-y)^{n-2})/y](i/\sigma^2)\frac{1}{2}\sigma_{AA}^2\} \\ &= (i/\sigma^2)\sigma_A^2 + (1-y)\{[(1-y)^{n-2}]/y\} - \{[(1-y)^{n-1}]/y\}(i/\sigma^2)\frac{1}{2}\sigma_{AA}^2 \\ &= (i/\sigma^2)[\sigma_A^2 + (1-y)^{n-1}\frac{1}{2}\sigma_{AA}^2],\end{aligned}$$

which is the expression previously found for the two-locus case.

Finally, it is necessary to evaluate the generalized term for differences in means on relaxation of selection,  $n\Delta\mu_{(m-1),m}$ , in terms of the two-locus example. To do this it is necessary to recast the general expression as follows:

$$\begin{aligned}n\Delta\mu_{(m-1),m} &= \sum_{ijklrstu} (f_{ik}^{n,m-2} f_{jl}^{n,m-2})(f_{rt}^{n,m-2} f_{su}^{n,m-2}) h_{(ik,jl)(rt,su)} \\ &\quad - \sum_{ijklrstu} (f_{ik}^{n,m-1} f_{jl}^{n,m-1})(f_{rt}^{n,m-1} f_{su}^{n,m-1}) h_{(ik,jl)(rt,su)} \\ &= A - B.\end{aligned}$$

where

$$(f_{ik}^{n,m-2} f_{jl}^{n,m-2}) = \text{frequency of the arbitrary sire } (A_i^1 A_k^2)(A_j^1 A_l^2) \text{ in } \Pi_{n,m-2},$$

$$(f_{rt}^{n,m-2} f_{su}^{n,m-2}) = \text{frequency of the arbitrary dam } (A_r^1 A_t^2)(A_s^1 A_u^2) \text{ in } \Pi_{n,m-2},$$

and

$$h_{(ik,jl)(rt,su)} = \text{mean of the full-sib array resulting from the cross}$$

$$[(A_i^1 A_k^2)(A_j^1 A_l^2) \times (A_r^1 A_t^2)(A_s^1 A_u^2)].$$

The mean of the full-sib array may be expanded as follows:

$$\begin{aligned}h_{(ik,jl)(rt,su)} &= [(1-y)/2]^2 (d_{ik,rt}^0 + d_{ik,su}^0 + d_{jl,rt}^0 + d_{jl,su}^0) \\ &\quad + \frac{1}{2}y[(1-y)/2] (d_{ik,ru}^0 + d_{ik,st}^0 + d_{jl,ru}^0 + d_{jl,st}^0) \\ &\quad + \frac{1}{2}y[(1-y)/2] (d_{il,rt}^0 + d_{il,su}^0 + d_{jk,rt}^0 + d_{jk,su}^0) \\ &\quad + (y/2)^2 (d_{il,ru}^0 + d_{il,st}^0 + d_{jk,ru}^0 + d_{jk,st}^0).\end{aligned}$$

The  $A$  term becomes,

$$A = \sum_{ijklrstu} \{f_{ik}^{0,0} f_{jl}^{0,0} f_{rt}^{0,0} f_{su}^{0,0} + n(i/\sigma^2) f_{ik}^{0,0} f_{jl}^{0,0} f_{rt}^{0,0} f_{su}^{0,0} (\alpha_i^1 + \alpha_j^1 + \alpha_r^1 + \alpha_s^1 + \alpha_k^2 + \alpha_l^2 + \alpha_t^2 + \alpha_u^2) \\ + (1-y)^{m-2} a(i/\sigma^2) f_{ik}^{0,0} f_{jl}^{0,0} f_{rt}^{0,0} f_{su}^{0,0} [\alpha_i^1 + \alpha_j^1 + \alpha_r^1 + \alpha_s^1 + \alpha_k^2 + \alpha_l^2 + \alpha_t^2 + \alpha_u^2] \\ + (\alpha\alpha)_{ik} + (\alpha\alpha)_{jl} + (\alpha\alpha)_{rt} + (\alpha\alpha)_{su}\} + (1-y)^{m-2} (b-n)(i/\sigma^2) f_{ik}^{0,0} f_{jl}^{0,0} f_{rt}^{0,0} f_{su}^{0,0} \\ \times (\alpha_i^1 + \alpha_j^1 + \alpha_r^1 + \alpha_s^1 + \alpha_k^2 + \alpha_l^2 + \alpha_t^2 + \alpha_u^2) \} h_{(ik,jl)(rt,su)}^0,$$

where

$$a = [(1 - (1-y)^n)/y],$$

and

$$b = \{n - [(1 - (1-y)^n)/y]\}.$$

Hence

$$A = [n + (1-y)^{m-2}(b-n) + (1-y)^{m-2}a](i/\sigma^2)\sigma_A^2 + [a(1-y)^{m-1}](i/\sigma^2)\frac{1}{2}\sigma_{AA}^2.$$

Likewise

$$B = [n + (1-y)^{m-1}(b-n) + (1-y)^{m-1}a](i/\sigma^2)\sigma_A^2 + [a(1-y)^m](i/\sigma^2)\frac{1}{2}\sigma_{AA}^2.$$

Then

$$n\Delta\mu_{(m-1),m} = A - B \\ = (1-y)^{m-1}[1 - (1-y)^n](i/\sigma^2)\sigma_{AA}^2,$$

as previously found.

In this way it is clear that the generalized analysis yields detailed results when the gene structure of the situation is specified.

### III. DISCUSSION

The main objective of this study is to attempt a generalized descriptive treatment of the consequences of truncation selection based on the individual phenotype. The generalization is possible by broadening the concept of the unit of inheritance to include not only genes but also gametes and individuals.

Analyses utilizing the notion of higher-order hereditary units with a gene interpretation based on the generalized Kempthorne gene model allow a detailed examination of the consequences of selection and relaxation following selection for a two-locus case. These analyses show that the immediate response to selection may be different from that predicted on the basis of the gene analysis if additive  $\times$  additive type of epistasis occurs. However, due to the "mutability" of these higher-order inheritance units, the population mean, on relaxation of selection, decays to that predicted by the gene analysis.

Some of the assumptions underlying the theory presented in this study should be emphasized because it is on the fulfilment of these assumptions that the accuracy of the analytical description of the response to selection rests.

First, it is assumed that the populations are infinite in size. In actual experiments, if *small* samples are taken to represent the populations, genetic "drift" will undoubtedly affect the reliability of the prediction procedure.

Second, it is assumed that the effects of individual genes are small so that the square and products of the quantities (gene effect/total phenotypic standard deviation) can be neglected. When more than one gene locus is involved it is assumed that the analysis deals, separately, with only small sub-sets of the total set of loci. In this way the approximations still hold. The total response is then obtained by an appropriate summing of responses over all such small sub-sets. However, as pointed out before, the errors introduced by the approximations tend to accumulate so that the basis of prediction becomes more subject to error as the mean of the selected population becomes farther removed from its original position.

The third point is that the theory developed in this study assumes that the reproductive value is the same for all selected genotypes. That is, natural selection is not operating differentially on the various genotypes. In this connection, probably the most interesting outcome of the study is that even if the assumption is true, the response to selection and relaxation of selection mimics the response which would occur if natural selection *were* operating antagonistically to artificial selection. For example, for the two-locus case in which natural selection is *not* operating and  $\sigma_{AA}^2$  is *not* equal to zero, it is found that the increment change due to selection in successive generations yields successively smaller increments. More explicitly, in the expression

$$\Delta\mu_{n,n-1} = (i/\sigma^2)[\sigma_A^2 + (1-y)^{n-1} \frac{1}{2}\sigma_{AA}^2],$$

it is clear that the value of the increment decreases as  $n$  increases. This diminishing of increments is somewhat similar to the effect that one would expect on the assumption that the intensity of natural selection increases as the cumulative change in the population mean due to artificial selection increases.

Likewise, the decay of the mean on relaxation from

$$\mu_{n,0} = (i/\sigma^2)[n\sigma_A^2 + \sum_{r=1}^n (1-y)^{r-1} \frac{1}{2}\sigma_{AA}^2]$$

to

$$\lim_{m \rightarrow \infty} (\mu_{n,m}) \rightarrow (i/\sigma^2)n\sigma_A^2$$

simulates the response which would occur if natural selection were operating in the absence of artificial selection to regress the mean toward the original unselected value. Thus, it would appear that in order to establish that natural selection is operating antagonistically to artificial selection, it would be advantageous to use criteria other than those mentioned above.

Finally, in the detailed case involving two linked loci, it is assumed that the recombination frequency is the same for the two sexes. This simplification often does not exist. For example, an extreme case occurs in *Drosophila* where crossing over does not occur in the male. Thus, a somewhat more complicated analysis is required to accommodate different recombination values in the two sexes. However, such a complication does not change the general picture; it merely alters the speed of the response to selection and response to relaxation following selection.

## IV. ACKNOWLEDGMENTS

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# SIMULATION OF GENETIC SYSTEMS BY AUTOMATIC DIGITAL COMPUTERS

## VII. EFFECTS OF REPRODUCTIVE RATE, AND INTENSITY OF SELECTION, ON GENETIC STRUCTURE

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### *Summary*

Simulation by Monte Carlo methods of the effect of selection against phenotypic extremes has shown that selection can produce a degree of genetic canalization which is more restrictive than that indicated by the limits of selection, showing that canalization of a rigid degree can be caused by loose selection.

### I. INTRODUCTION

This paper is a direct extension of Part VI of this series (Fraser 1960), describing further runs of the "epistasis" programme. In the initial runs of this programme the rate of reproduction was maintained constant at 50 progeny per mating, and the criteria of selection was similarly maintained constant at  $\pm 1.0$ . In these previous runs, variations of the structure of the genetic system, and of the number of parents, were compared, showing that selection against phenotypic extremes would lead to the fixation of a simple additive genetic system at an extremely slow rate in all but very small populations. With the epistatic systems defined by this programme such selection operated to modify the relation of genotype to phenotype, which in this particular system became an S-shaped function. The efficiency of selection in these runs was independent of population size.

In the further runs of this programme described below, two aspects have been examined. These are (i) the effect of variation of the number of progeny per mating, and (ii) the effect of variation of the intensity of selection.

### II. PARAMETERS

Although the epistasis programme has been constructed to minimize the number of parameters maintained constant during a run, some must be specified. These are:

- (1) The type of mating—self-fertilization or random mating.
- (2) The number of parents—this has been set to 160 per generation for all the present runs.
- (3) The reproductive rate—this has been set at 10, 20, 30, 40, and 50 progeny per mating, in two independent sets of runs, which are termed the "reproductive rate" set.

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- (4) The maximum and minimum limits of eligible phenotypes—these have been set at  $\pm 1.0$ ,  $\pm 2.0$ ,  $\pm 3.0$ ,  $\pm 4.0$ ,  $\pm 5.0$  respectively, in two independent sets of runs, termed the "selection intensity" set.
- (5) The vectors of coefficients of dominance and interaction—these have been set at the ranges used in the previous runs (see Fraser 1960), allowing considerable potential variation of both dominance and epistasis.

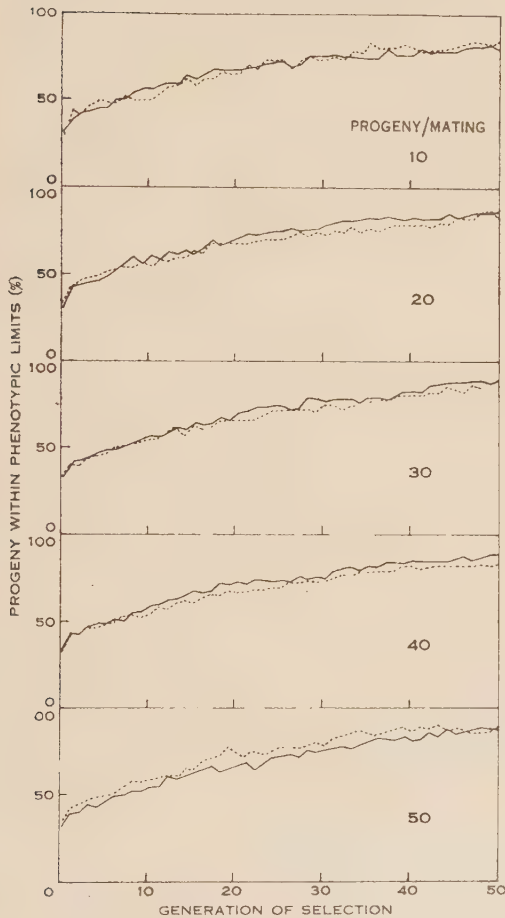


Fig. 1

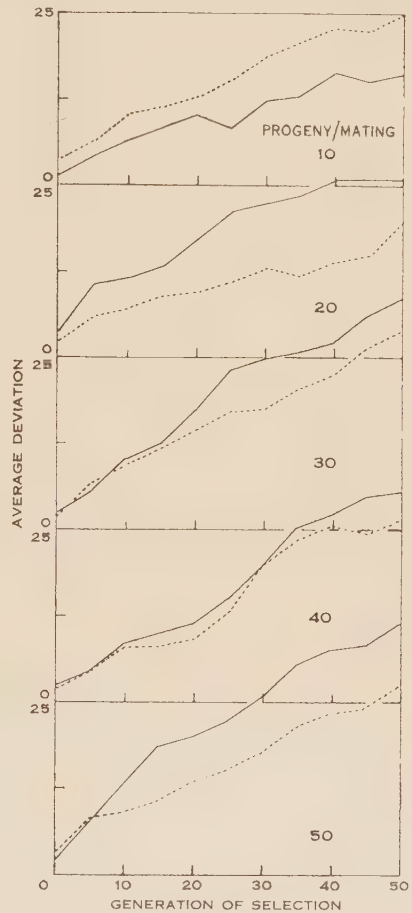


Fig. 2

Fig. 1.—Percentage of progeny with phenotypes within selection limits plotted against generation of selection, for the reproductive rate set of runs.

Fig. 2.—Average deviation of the gene frequencies of the basic loci from the initial value of 0.5 at which the runs were started.

The two sets of runs involving different numbers of progeny per mating were all made with the same criteria of selection, namely  $\pm 1.0$ . The two sets of runs involving different criteria of selection were all made at a reproductive rate of 10 progeny per mating.

## III. RESULTS AND DISCUSSION

## (a) Reproductive Rate

The results from the reproductive rate set of runs are illustrated in Figures 1 and 2. Figure 1 shows the percentage of progeny which had phenotypes within the selection limits plotted against generation of selection. This measures the efficiency of selection. Any differences between runs made at different numbers of progeny

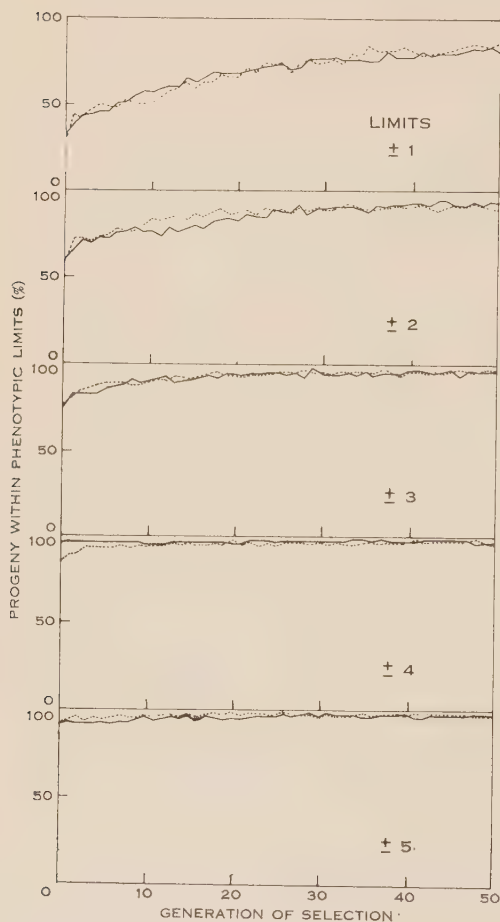


Fig. 3

Fig. 3.—As for Figure 1, for the selection intensity set of runs.

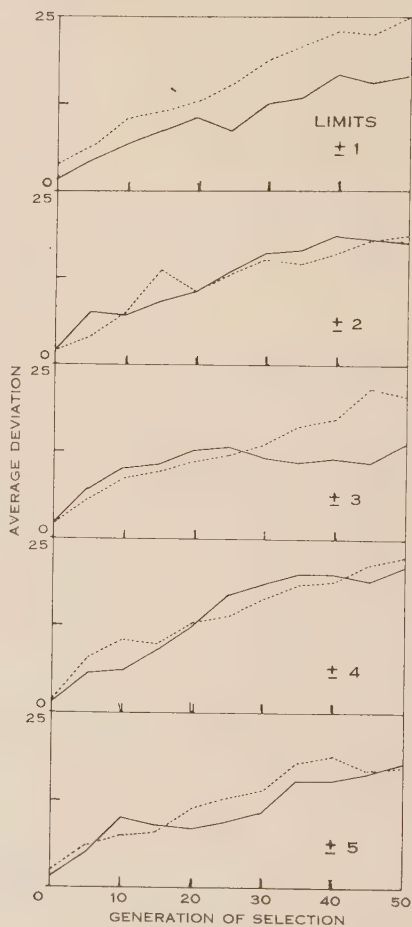


Fig. 4

Fig. 4.—As for Figure 2, for the selection intensity set of runs.

are small, and justify the conclusion that selection against phenotypic extremes is not markedly affected by population size, where there is available variation of the epistatic component of genetic variation.

Figure 2 shows the average deviation of the gene frequencies of the basic loci from the initial frequency of 0.5, at which the runs were started (Fraser 1960).

Again, the differences between runs are small, apart from those made at 10 progeny per mating, which show a markedly slower rate of increase of deviations from the initial gene frequency. Since one of the most interesting features of selection against extremes is its effect on the distribution of gene frequencies of the "basic" additive loci, it follows that the parameters should be set to minimize the effects of population size, reproductive rate, etc. Therefore, in the selection intensity set of runs the reproductive rate has been set at 10; the population size has been set at 160, the maximum possible for this programme.

(b) *Selection Intensity*

The results from the selection intensity set of runs are shown in Figures 3-7. Figure 3 shows the percentage of progeny which had the phenotypes within the selection limits plotted against generation of selection.

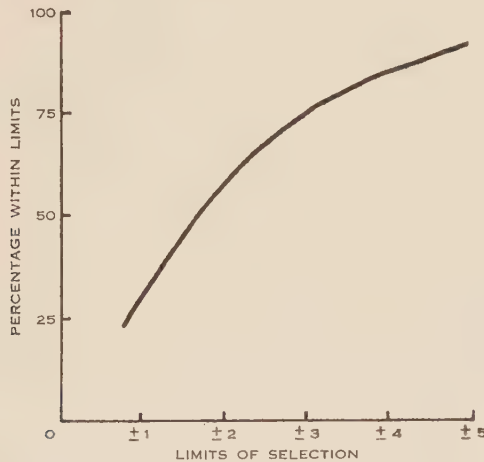


Fig. 5.—Percentage of progeny with phenotypes within limits plotted against selection limits, for the first generation, prior to selection.

It is clear, as would be expected, that the efficiency of selection, as measured by the percentage of progeny with phenotypes within the selection limits, is positively correlated with the intensity of selection, this being low for wide selection limits, high for narrow selection limits. The percentage of progeny in the first generation of selection which are within the selection limits is plotted against selection limit in Figure 5.

In the previous paper the conclusion was drawn that selection against extremes is a minor contributor to the incidence of genetic fixation of a complex epistatic genetic system. This conclusion is verified in the present set of runs. Figure 4 shows the average deviation of gene frequencies from the original value of 0.5 plotted against generation of selection for the selection intensity set of runs.

There are no marked differences between the runs at different selection limits in the rate of increase of deviations from the original gene frequencies. Consequently,



it is safe to conclude that the trend towards genetic fixation is predominantly controlled by the population parameters of reproductive rate and number of parents.

The marked reduction of phenotypic variation produced by selection in these runs is due to a change of the relation of genotype-phenotype from a linear (additive) function to an S-shaped (epistatic) function. This change is measured, in this programme, by the  $Q$  and  $C$  subgenotypes, which determine the values of the coefficients of the function relating genotype to phenotype. This function is

$$P = x + qx^2 + cx^3,$$

where  $P$  is the phenotype, and  $x$  is the additive+dominance component of the genotype.

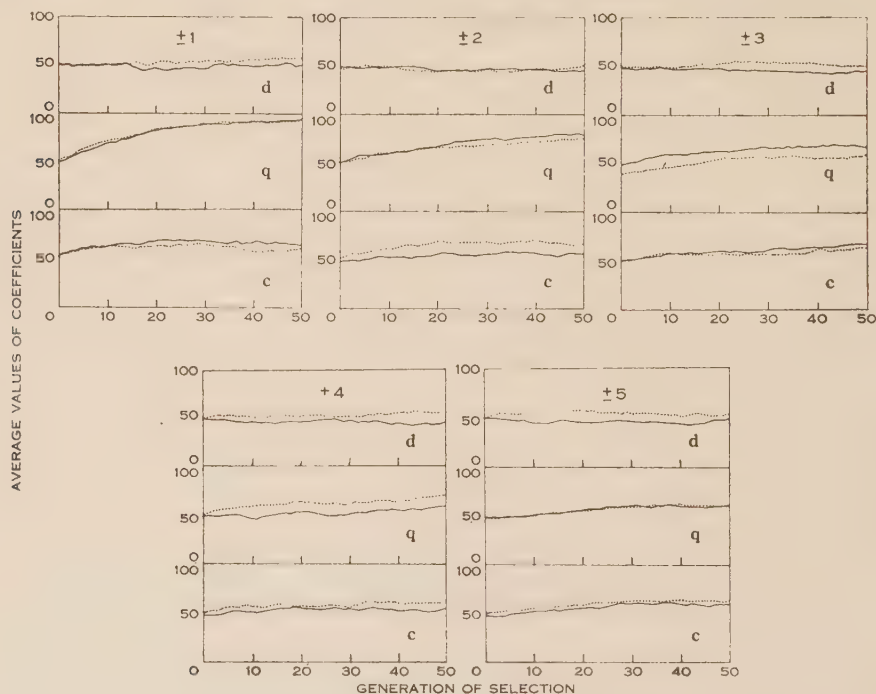


Fig. 6.—Average values of the  $d$ ,  $q$ , and  $c$  coefficients plotted against generations of selection, for the selection intensity set of runs.

The effects of selection on  $q$  and  $c$  are shown in Figure 6, where the average values of these coefficients are plotted against generation of selection. These coefficients can be used to calculate the genotype-phenotype relationships. These are shown in Figure 7, calculated from the values of  $q$  and  $c$  at the 50th generation of selection.

An interesting feature of these results is that selection against phenotypic extremes produces a greater reduction of the phenotypic variance than is indicated from the limits of selection. This is particularly evident from the comparisons of

the genotype-phenotype relation produced by selection at limits of  $\pm 2.0$ , with those produced by selection at limits of  $\pm 3.0$ ,  $\pm 4.0$ , and  $\pm 5.0$  respectively. The latter determine a lesser phenotypic variance even though produced by less intense selection.

A reason for this apparently exaggerated response to selection can be found in the determination of  $q$  and  $c$ . These are each determined by segregating subgenotypes. Consequently, the specific values of the genotype-phenotype relation shown in Figure 7 are mean values, with ranges given by the segregation of the  $Q$  and  $C$  subgenotypes. Selection will operate against the extreme values of the  $q$  and  $c$  coefficients, causing a greater degree of "canalization" than is expected from measurements of reproductive fitness.

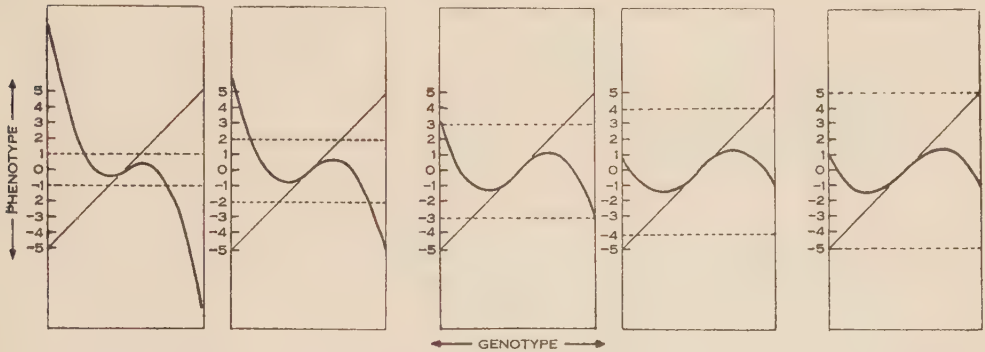


Fig. 7.—Relationship of genotype-phenotype determined by the values of the  $Q$  and  $C$  subgenotypes, at the 50th generation of selection.

These results from simulated genetic systems are analogous to those obtained in analyses of vibrissa number in mice (Fraser and Kindred 1960). This character is fairly strongly canalized at a vibrissa number of 18–19. Mice have been produced in selection experiments with numbers both above and below this value. On the assumption that the canalization is an effect of selection against extreme vibrissa numbers, measurements were made of various components of reproductive fitness on a wide range of mice with different vibrissa numbers. Only mice with very low or very high vibrissa numbers showed a decreased fitness. This indicated that direct selection on vibrissa number was not very intense, although it is possible that vibrissa number has effects on aspects of reproductive fitness which were not measured in these experiments, i.e. the number of vibrissae is an important characteristic under conditions which, because they do not occur in an experimental mouse colony, are not measured. Conversely, Rendel (personal communication) considers that the most probable reason for canalization of an external character is that due to the necessity for maintenance of a specific pattern of development of the tissue concerned, i.e. number of vibrissae is maintained constant at 18–19 as a secondary consequence of the regularity of development of all epithelium. Selection against such irregularities would be intense. These hypotheses both provide a possible explanation of the apparent lack of intense selection against extremes of

a "canalized" character, one on the direct effects of the character on some unmeasured aspect of fitness, the other on a "pleiotropic" relationship of the character to other more important characters which are under intense selection. The results obtained in the selection intensity set of runs show that neither explanation is necessary, since selection operative at limits far removed from a "canalization zone" can cause a sufficient reduction of phenotypic variance.

An interesting feature of the form of the genotype-phenotype relation is that selection against extremes has modified this from the linear additive form to a sigmoid form which has an inverse direction to that of the linear form. Genetic substitution would, in the presence of the sigmoid function, produce an effect opposite to that produced by the same genetic substitution in the presence of the linear function. This is due to the genetic model being based on a cubic equation determining the form of the genotype-phenotype relation. It is possible, but not certain, that a model based on a higher-order equation would not show this unexpected divergence between additive and epistatic systems. An experimental test is possible where the basic genetic system has pleiotropic effects on a canalized and on a non-canalized character. Response to selection should then be negatively correlated between the two characters.

#### IV. ACKNOWLEDGMENT

The author is indebted to Miss C. Jones for her help in preparing the diagrams.

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# GROWTH OF THE MOUSE COAT

## VII. HAIR CYCLES AND SEBACEOUS GLANDS IN HOMOZYGOUS AND HETEROZYGOUS NAKED MICE

By T. NAY\*

[Manuscript received March 4, 1960]

### *Summary*

The action of the Naked gene ( $N$ ) in homozygous mice has additive effects on keratinization and sebaceous glands but not on the timing of hair cycles.

### I. INTRODUCTION

The Naked gene ( $N$ ) appeared as a spontaneous mutation in an albino stock of normal mice and was first described by Lebedevsky and Dauvert (1927), and subsequently by David (1931, 1932), Snell (1931), Redlichs (1937*a*, 1937*b*), Ebenhorst Tengbergen (1939), F. C. Fraser (1946), Steinberg and F. C. Fraser (1946), and Danneel and Kahls (1947).

A method for recording and measuring the time intervals between consecutive hair cycles in Naked mice has been described in detail in Parts II-V of this series (cf. A. S. Fraser and Nay 1953, 1955; Nay and A. S. Fraser 1954, 1955). In this Part the time intervals between consecutive hair cycles in homozygous ( $NN$ ) and heterozygous ( $N-$ ) Naked mice is compared and the effect of the  $N$  gene on the development of the sebaceous glands in the two genotypes is described.

### II. MATERIALS AND METHODS

Three groups of mice were used for the comparison of the time interval between consecutive hair cycles in the two genotypes:

- (1) Ten homozygous Naked mice ranging in age between 63 and 370 days and taken from a heterogeneous stock segregating for the Naked gene.
- (2) Ten heterozygous Naked mice from the same stock.
- (3) Ten heterozygous Naked mice from NA stock, a highly inbred subline of the strong A strain which has been kept segregating for the  $N$  gene.

For two months the positions of the hair bands were determined along a line running from head to tail along the side of the animal, and recorded on a series of drawings. The line was divided into 64 units. The data were plotted on graphs against time, and intervals (in days) between consecutive hair cycles for positions 20, 30, 40, and 50 measured.

For the study of sebaceous glands,  $++$ ,  $N+$ , and  $NN$  mice from the heterogeneous stock were used, and both whole mounts and paraffin sections of the sebaceous gland were studied. For durable whole mounts the technique of Quay (1954) was used with some modifications as described below. The mice were skinned

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immediately after killing, the skin pinned to a rectangular cork frame, and fixed for at least 24 hr in 5 per cent. formol-saline. After washing in running water for c. 2 hr, pieces of skin  $1\frac{1}{2}$ –2 cm wide and 3–5 cm long and covering the area between the nape of the neck and the tail root were cut off, shaved, and placed in staining solution in a covered petri dish. The staining solution was prepared from 30 ml 0.5 per cent. oil blue N in isopropanol which was diluted with 20 ml distilled water. After 6 hr in staining solution the pieces of skin were taken out and cleaned on both sides with a towel to remove precipitated oil blue particles and other impurities. Subsequently, the pieces of skin were mounted in glycerol, the epidermal surface up, and examined under the microscope. The sebaceous glands and sebum were stained dark blue.

TABLE 1  
MEAN VALUES FOR THE TIME INTERVAL (IN DAYS)  
BETWEEN CONSECUTIVE HAIR CYCLES IN THE TWO  
GENOTYPES

Position	Time Interval (days)		
	NN	N + *	N + †
20	19.8	20.8	21.3
30	20.8	22.6	22.6
40	22.9	23.5	23.7
50	23.8	23.9	25.5

\* Mice from same stock as homozygous mice.

† Mice from an inbred, unrelated stock.

For the preparation of paraffin sections the skins from freshly killed animals were pinned to rectangular cork frames and fixed in 5 per cent. formol-saline. After washing, the skins, still pinned to the frames, were passed through 70 per cent., 95 per cent., and two changes of absolute alcohol (c. 30 min in each) and then cleared in xylol. After clearing, the skins were infiltrated with paraffin (two changes, c. 24 hr in each) and rectangular pieces c.  $1\frac{1}{2}$ –2 cm wide and c. 3 cm long were cut out and embedded in paraffin-beeswax (5 per cent.) mixture. Serial sections, 10  $\mu$  thick, were stained for 30 sec in 0.2 per cent. polychrome methylene blue. After staining and rinsing for a short time in tap water, the stain was fixed in equal parts of 5 per cent. ammonium molybdate and 1 per cent. potassium ferrocyanide for 5 min, washed again in tap water, passed through graded alcohols, cleared in xylol, and mounted in Canada balsam.

Photomicrographs of both whole mounts and serial sections were taken when necessary.

## III. RESULTS

(a) *Hair Cycles*

The average values of the time intervals between consecutive hair cycles for each of the four positions for the three groups of mice is given in Table 1. It was shown by *t*-tests that the intervals in *N* + and *NN* genotypes do not differ significantly.

(b) *Sebaceous Glands*

In the normal mouse the sebaceous glands join the follicles well below the surface of the skin (Plate 1, Fig. 1). The ducts are short and there is no accumulation of sebum in the hair canal above the gland. There might be, in catagen or early telogen, accumulation of sebum below the sebaceous gland (Plate 1, Fig. 4). Unilobal glands could be found occasionally, but they are rather exceptional.

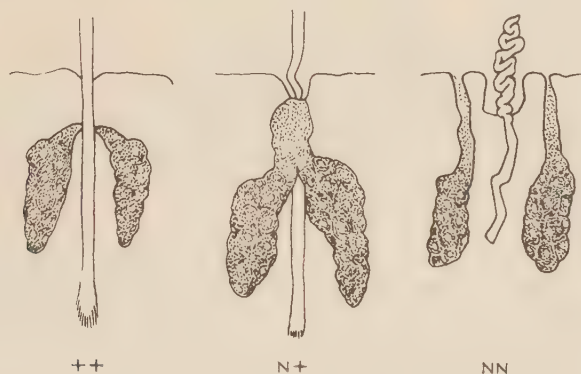


Fig. 1.—Diagrammatic representation of typical sebaceous glands in ++, *N* +, and *NN* genotypes. ++, normal sebaceous gland: the ducts are short and join the hair follicle separately. *N* +, sebaceous gland in *N* + genotype: the ducts are joined and form an ampulla. *NN*, sebaceous gland in *NN* genotype: both ducts are separated from the hair follicle and open independently on the surface of the skin.

The sebaceous glands in the heterozygous Naked mouse differ from those in the normal mouse in many respects. The lobes were much larger, and the ducts were longer and wider, and were fused together, forming an ampulla round the hair fibre. The ampulla and the extended ducts were filled with sebum. Unilobal glands were frequent (Plate 1, Fig. 2).

In homozygous Naked mice the sebaceous gland ducts may fuse and form a wide ampulla immediately under the surface of the skin, or, as happens in most cases, the two ducts do not join but open on the surface of the skin independently, by-passing the hair canal (Plate 1, Fig. 3). The ducts were tortuous, passing below or round the follicles before they reach the surface of the skin. The hair canal itself is extended and contains a crumpled, rudimentary hair. The hair may be a corkscrew-like mass of keratin, which is caked together and extends out above the skin (Plate 1, Fig. 5).

Types of glands, characteristic for each genotype, are presented diagrammatically in Figure 1.

#### IV. DISCUSSION

There are three most obvious effects of the Naked gene on the hair follicle:

- (1) Impaired keratinization.
- (2) Abnormal sebaceous glands.
- (3) Timing of follicle activity.

According to David (1931), the hairs in heterozygous Naked mice break off because of faulty keratinization. The action of the *N* gene on fibre formation is strongly additive in the homozygous condition: in *N*+ mice the process of keratinization fails shortly before the completion of the hair growth, whereas in the *NN* genotype the same process breaks down to such an extent that the animals never produce a coat.

The differences in sebaceous glands between the two genotypes are but one aspect of the disorganization of the hair follicle by the Naked gene. David (1931) found that the hair follicles were smaller in *N*+ than in + mice, and still smaller in the *NN* genotype. It seems that the *N* gene causes the deficiency of an agent necessary to produce a functionally efficient hair follicle. In *N*+ animals this deficiency is partly compensated by the presence of the +locus, whereas in the *NN* genotype the gene action is expressed fully.

As shown in Table 1, there is no real difference in the time interval between consecutive hair cycles between the two genotypes (differences are so small as to be statistically insignificant). There are reasons to believe that the regular recurrence of hair cycles in Naked mice is caused by the lack of an inhibitor, which may operate in adult normal mice. Hair cycles in Naked mice become inhibited in conditions such as pregnancy and lactation. The follicles, unchecked, would regrow regularly after a certain period of time, which seems to be constant but differing in length according to the position on the body. Such a view may be supported by the fact that there is no additive action of the *N* gene on the time interval between consecutive hair cycles in homozygous conditions.

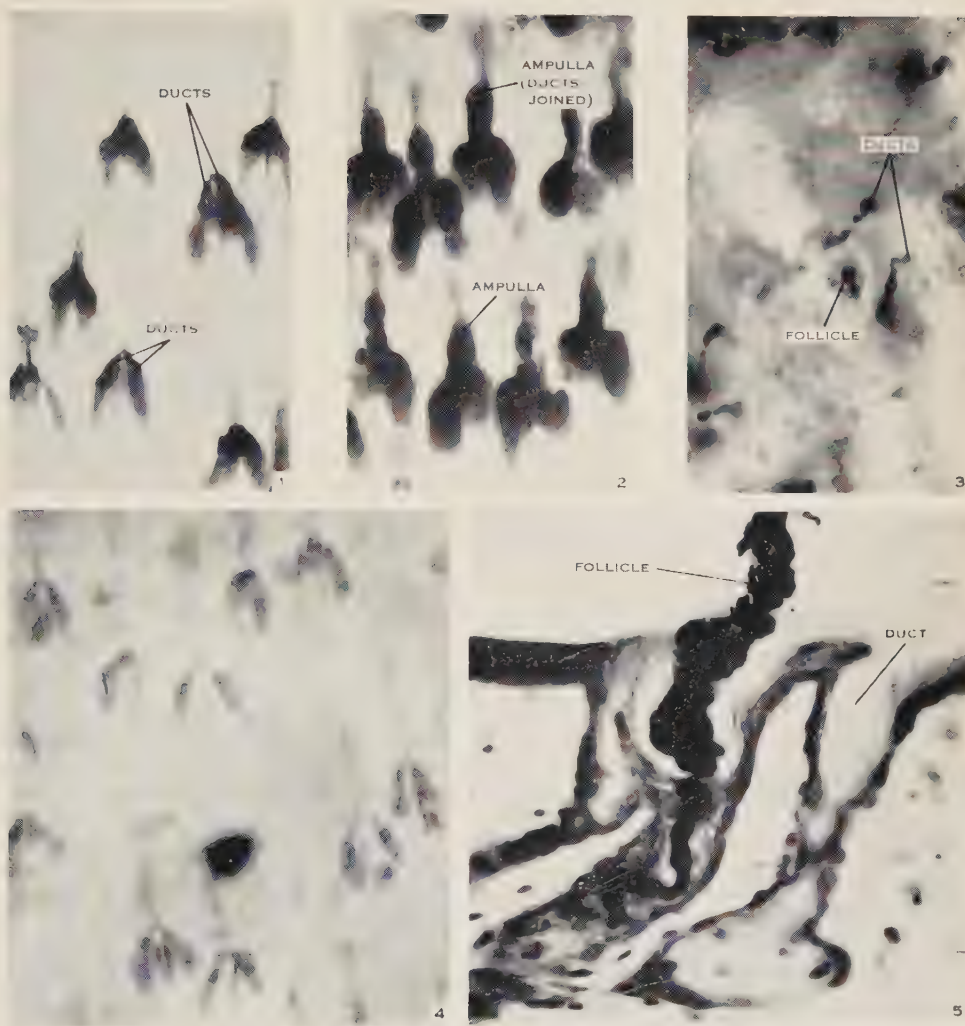
#### V. ACKNOWLEDGMENTS

The author wishes to thank Dr. J. M. Rendel and Dr. A. S. Fraser for advice and criticism of the manuscript, and also Mr. K. M. Adams for photographic assistance.

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#### EXPLANATION OF PLATE I

Photomicrographs of sebaceous glands in ++, N+, and NN genotypes. Figures 1–4: whole mounts, stained in oil blue N

Fig. 1.—Normal sebaceous gland: the ducts are short and join the hair follicle separately.

Fig. 2.—Sebaceous gland in N+ genotype: the ducts fuse and form an ampulla.

Fig. 3.—Sebaceous gland in NN genotype: both ducts are separated from the hair follicle and open wide apart on the surface of the skin.

Fig. 4.—Normal sebaceous glands: sebum in hair canal below the sebaceous glands.

Fig. 5.—Sebaceous gland in NN genotype in side view. Paraffin section, stained in polychrome methylene blue.

# URINARY EXCRETION OF CREATINE IN THE SHEEP

By E. BLANCH\* and B. P. SETCHELL\*

[*Manuscript received March 21, 1960*]

## *Summary*

Creatinuria was found in normal rams, wethers, and ewes on ordinary diets. This finding emphasizes the need for caution in interpreting urine creatine concentration or creatine to creatinine ratios used as diagnostic aids in muscular dystrophy in sheep. No change in creatine output per 24 hr was observed when the ewes were fasted for 6 days. Values are given for the ratio creatine clearance to creatinine clearance in rams, wethers, and ewes and for creatine clearance in ewes.

## I. INTRODUCTION

Two early reports of Lindsay (1912) and Orr (1918) described the presence of creatine in the urine of fed sheep, but Palladin (1924) found it only in the urine of fasting animals. Hunter (1928) explained these results and his own (Hunter 1914) by suggesting that the fed sheep were actually losing condition. Green (1918) also found creatine in the urine of fed sheep.

Creatinuria is found in cases of human muscular dystrophy and Whiting, Willman, and Loosli (1949), Bacigalupo *et al.* (1952), Draper, James, and Johnson (1952), and Hartley (1953) found creatine in the urine of lambs with muscular dystrophy—both naturally occurring cases and cases induced by artificial diets, and it seemed that the presence of creatine in urine may have been of diagnostic value in this condition in lambs. Although some of these authors quoted urine creatine concentrations in lambs which were fed the artificial diets with added *dl-a*-tocopherol and which did not develop muscular dystrophy, none of them quoted values from normal lambs on ordinary diets. In preliminary investigations, using the same method as the earlier workers, the present authors found that the urine of lambs from a few weeks of age onwards and of adult sheep contained a substance reacting like creatine. This method is notoriously subject to error, and the present work, using a method more specific and reliable than those available at the time of these early reports, was undertaken to clarify these differences.

## II. MATERIALS AND METHODS

Aged Corriedale ewes and wethers were fed oaten chaff (diet B, 4 per cent. protein), and two-tooth Corriedale × Merino rams and one aged Dorset ram were given mixed feed consisting of (by weight) 40 per cent. lucerne chaff, 40 per cent. oaten chaff, 10 per cent. bran, and 10 per cent. crushed oats with fine salt added to 0.1 per cent. (diet A, 8 per cent. protein). Water was allowed to all groups freely throughout.

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Ewes were catheterized and the urine collected directly into chilled (crushed ice) receivers, 5 minutes' collection usually being sufficient. Rams and wethers, however, cannot be catheterized directly, and urine from these animals was collected by suffocating them until urine was passed; a portion was then transferred to the ice-bath. The samples were maintained at 0°C until applied to chromatography paper (no longer than 1–2 hr). Blood samples were collected from the jugular vein into heparin.

Urinary creatine was estimated quantitatively by the method of Eden, Harrison, and Linnane (1954), which involves paper chromatographic separation of creatine from interfering related compounds. The optical densities of the final solutions were read on a Beckman model DU spectrophotometer. 10–20  $\mu$ l (multiples of 5  $\mu$ l with drying between applications) was a convenient urine volume to use. The solvent (75 per cent. aqueous ethanol) was allowed to run 7–9 hr at room temperature. Each urine sample was checked for the presence of  $\alpha$ -naphthol-diacetyl-reacting substances other than creatine by cutting the dried papers into 2-cm strips and dipping these into the reagent as described by Eden, Harrison, and Linnane. Similar  $R_F$  values for creatine were obtained. In no case was there any spot other than that corresponding to known creatine. The method was also tested with normal male adult human urine, which is generally considered creatine-free: no creatine was present (five samples). Creatinine gave a slow positive reaction at  $R_F$  0.66 by virtue of its conversion to creatine (verified by alkaline picrate).

Plasma creatine was determined as by Eden, Harrison, and Linnane but with corrections for mono- and unsubstituted guanidines (Setchell, unpublished data 1960).

Plasma and urinary creatinine concentrations were obtained by the alkaline picrate method as modified by Hare (1950) with the use of Lloyd's reagent.

### III. RESULTS

Creatine was present in considerable amounts in the urine of all the groups (Table 1), and in the wethers only was the output of creatine less than that of creatinine. The higher concentration of creatine in the urine of the ewes after 6 days of fasting is due to diminished urine volume: the creatine excretion per day in this group was similar before and after fasting. The creatine to creatinine renal clearance ratios indicate that about 70 per cent. of creatine passing the glomerulus was resorbed by the tubules, assuming creatinine clearance is a measure of glomerular filtration rate in the sheep (Shannon 1937; Schmidt-Nielsen *et al.* 1958).

### IV. DISCUSSION

The present findings of creatinuria in sheep on a normal diet confirms those of Lindsay (1912) and Orr (1918) and contradict those of Palladin (1924). In any case, the agreement in Palladin's paper of the creatinine values before and after autoclaving (the difference assumed to be due to creatine) is so good as to be almost incredible. The values for the ratio of urinary creatine to creatinine are even higher with the present method than older values, calculated from the literature and summarized in Table 2, for sheep and some other ruminants.



TABLE 1  
URINE AND PLASMA CREATININE AND CREATININE CONCENTRATIONS AND CLEARANCES IN THE SHEEP  
Values given are means and standard errors of the means

No. of Sheep and Diet	(C) Creatine Concn. in Urine ( $\mu\text{g/ml}$ )	Amount of Creatine Excreted in 24 Hr (g)	Creatine Concn. in Plasma ( $\mu\text{g/ml}$ )	(D) Creatine Clearance (ml/min)	(E) Creatinine Concn. in Urine ( $\mu\text{g/ml}$ )	Amount of Creatinine Excreted in 24 Hr (g)	Creatinine Concn. in Plasma ( $\mu\text{g/ml}$ )	(F) Creatinine Clearance (ml/min)	Ratio D/F	Ratio C/E
5 rams, diet A	1060 $\pm$ 330	—	26.0 $\pm$ 2.1	—	678 $\pm$ 200	—	6.4 $\pm$ 0.7	—	0.39 $\pm$ 0.12	1.56 $\pm$ 0.15
5 wethers, diet B	660 $\pm$ 340	—	24.2 $\pm$ 0.9	—	1220 $\pm$ 340	—	7.6 $\pm$ 0.2	—	0.22 $\pm$ 0.03	0.72 $\pm$ 0.12
4 ewes, diet B	620 $\pm$ 140	1.10 $\pm$ 0.21	26.2 $\pm$ 1.1	32.5 $\pm$ 8.9	560 $\pm$ 60	0.99 $\pm$ 0.08	6.8 $\pm$ 0.3	109.9 $\pm$ 9.7	0.29 $\pm$ 0.06	1.2 $\pm$ 0.3
4 ewes, fasted 6 days	2450 $\pm$ 290	1.16 $\pm$ 0.14	30.1 $\pm$ 0.2	29.3 $\pm$ 1.2	1775 $\pm$ 260	0.84 $\pm$ 0.13	7.3 $\pm$ 0.4	82.9 $\pm$ 11.0	0.36 $\pm$ 0.03	1.5 $\pm$ 0.1

The occurrence of creatine in normal sheep urine may be simply a reflection of the higher plasma creatine concentration in sheep than in man: 24-30  $\mu\text{g/ml}$  compared with 6.6 and 8.1  $\mu\text{g/ml}$  for two samples of pooled human plasma by the present method and less than 6  $\mu\text{g/ml}$  for human males by autoclaving and alkaline picrate (Tierney and Peters 1943).

That creatinuria is a normal occurrence for sheep emphasizes the need for caution in interpreting urine creatine concentrations or creatine to creatinine ratios used as diagnostic aids in muscular dystrophy.

TABLE 2  
URINARY CREATINE TO CREATININE RATIOS FOR SOME RUMINANTS  
Ratios quoted are means and standard errors of the means

Animal	Sex	Fed or Fasted	Creatine/ Creatinine Ratio	No. of Observations	Reference
Sheep	?	Fed	$0.88 \pm 0.16$	4	Lindsay (1912)
	Female	Fed	$0.70 \pm 0.22$	2	Orr (1918)
	Female	Fasted	$0.80 \pm 0.13$	13	Hunter (1914)
	Wether	Fed	$0.58 \pm 0.08$	15	Green (1918)
	Wether	Fed	$0.0 \pm 0.0$	11	Palladin (1924)
	Wether	Fasted up to 6 days	Up to 1.87	25	Palladin (1924)
Goat	Male	Fed	0.91	1	Orr (1918)
	Female	Fed	$0.61 \pm 0.11$	2	Orr (1918)
Cattle	Male	Fed	$0.55 \pm 0.29$	4	Lindsay (1912)
	Female	Fed	$0.90 \pm 0.32$	4	Lindsay (1912)
	?	Fed	$0.69 \pm 0.04$	30	Nagy (1935)
(Calves)	?	Fed	$0.72 \pm 0.12$	12	Hart <i>et al.</i> (1911)
Camel	Female	Fed	$0.45 \pm 0.14$	5	Smith and Silvette (1928)
Dromedary	Male	Fed	$0.32 \pm 0.06$	2	Smith and Silvette (1928)
Llama	Male	Fed	$0.37 \pm 0.20$	2	Smith and Silvette (1928)
Alpaca	Male	Fed	0.68	1	Smith and Silvette (1928)

Our results also differ from those of Palladin (1924) in the effect of fasting on the creatine excretion, and this may possibly be due to body condition, animals in good condition probably drawing on fat reserves before protein.

The urinary creatine to creatinine ratio in the wethers was significantly less than that for the ewe and ram groups ( $P < 0.01$ ). This may be related to the observations of Wilkins and Fleischmann (1945), who found that methyltestosterone enhances creatine synthesis in the human in some way unrelated to its role as a methyl donor, even though these workers were unable to demonstrate methyltestosterone-induced creatinuria in laboratory animals and in pigs.

## V. ACKNOWLEDGMENT

It is a pleasure to acknowledge the technical assistance of Mr. R. Layland.

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# THE TOXICITY OF SOME ANTIBACTERIALS TO FOWL SPERMATOZOA

By R. G. WALES\* and I. G. WHITE\*

[*Manuscript received November 23, 1959*]

## *Summary*

In a study of the effect of a number of antibacterials on fowl spermatozoa, penicillin and sulphanilamide at 1 mg/ml, chloromycetin at 0.2 mg/ml, and terramycin at 0.5 mg/ml depressed motility at a 1 in 20 dilution of semen in a medium having a similar tonicity to fowl seminal plasma.

Streptomycin, sulphamezathine, aureomycin, and tetracycline were not significantly toxic in the highest concentrations used.

Penicillin, sulphanilamide, chloromycetin, and terramycin were less toxic when the semen was diluted 1 in 3 with Ringer or 1 in 20 with 30 per cent. seminal plasma instead of Ringer. This suggests the presence of a protective substance in the seminal plasma.

The toxicity of sulphanilamide and chloromycetin was not antagonized by *p*-aminobenzoic acid or phenylalanine respectively. The spermicidal effect of biotin, however, was reduced by aureomycin.

Oxygen uptake, fructolysis, and lactic acid production were consistently depressed by sulphanilamide at 5 mg/ml. A similar concentration of chloromycetin almost completely inhibited oxygen uptake although aerobic fructolysis and lactic acid production were unaffected. Metabolism was little affected by as much as 20 mg/ml penicillin.

## I. INTRODUCTION

Antibacterials have been used extensively in the preservation of bull semen for artificial insemination (see Emmens and Blackshaw 1956) and White (1954) has investigated the toxicity of a number of antibacterials for bull, ram, rabbit, and human spermatozoa. The most striking feature of these studies was the resistance of mammalian spermatozoa to bacteriostatic concentrations of antibacterials. Very high concentrations, however, of all but penicillin proved toxic to the spermatozoa of one or more species.

Fowl semen collected by abdominal massage (Burrows and Quinn 1939) is likely to be contaminated by excreta and has a higher initial bacterial content than bull semen (Wilcox and Shorb 1958). Smith (1949) reported that sulphathiazole, but not streptomycin, was toxic at 0.05 mg/ml, and more recently Wilcox and Shorb (1958) found that terramycin, tetracycline, and chloromycetin at a concentration of 0.9 mg/ml depressed the motility of fowl spermatozoa after 24 hr.

This paper compares the effect of a number of antibacterials on the motility and metabolism of fowl spermatozoa.

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## II. MATERIALS AND METHODS

*(a) Techniques*

Fowl semen was collected by abdominal massage (Burrows and Quinn 1939). Only normal uncontaminated pooled ejaculates of good initial motility were used.

Semen was usually diluted 1 in 20 for motility observations and 1 in 3 for metabolic studies. The tubes for motility observations were kept at room temperature and scored at hourly intervals by the system of Emmens (1947). Full motility was rated as 4 and complete immotility as 0. As quarter grades were frequently used the actual scores have been multiplied by 4, and the total score  $\times 4$  over the experimental period, the motility index, has been used as unit observation in the analyses of variance.

Metabolic studies were undertaken at 25°C. Oxygen uptake was measured over 3 hr by the direct Warburg technique (Umbreit, Burris, and Stauffer 1949). The shaking rate was 114 strokes/min and the gas phase air. For anaerobic experiments 1 ml of diluted sperm suspension was pipetted into a narrow tube, covered with 2 cm of oxygen-free paraffin, and stoppered. Fructose (Mann 1948) and lactic acid (Barker and Summerson 1941) were estimated at the start and end of both aerobic and anaerobic experiments.

After the metabolic experiments, total counts of spermatozoa were made in duplicate using a haemocytometer. Oxygen uptake, fructose utilization, and lactic acid production (per  $10^8$  cells) during the experimental period were then calculated and used as unit observation in the analyses of variance.

*(b) Diluents and Antibacterials*

The diluent of pH 7.0 used in most motility experiments consisted of 0.0136M  $\text{Na}_2\text{HPO}_4$ , 0.0064M  $\text{NaH}_2\text{PO}_4$ , 0.005M KCl, 0.0015M  $\text{MgCl}_2$ , 0.0641M NaCl, 0.25M glucose.

The diluent for metabolic and some motility studies was a modification of Ringer's solution and had the following composition: 0.01M  $\text{Na}_2\text{HPO}_4$ , 0.005M KCl, 0.001M  $\text{KH}_2\text{PO}_4$ , 0.001M  $\text{MgSO}_4$ , 0.1848M NaCl containing 100 mg per cent. fructose.

Both of these diluents have a relative tonicity of 140 (0.9 per cent. NaCl  $\equiv$  100 per cent. tonicity) which is close to the tonicity of fowl semen and optimal for motility in the presence of magnesium and potassium.

The antibacterials used were sulphanilamide (Drug Houses of Australia Ltd.), sulphamezathine (I.C.I.A.N.Z.), penicillin G, crystalline (Glaxo Laboratories Ltd.), streptomycin sulphate (Glaxo), chloromycetin (Parke, Davis and Co. Ltd.), aureomycin hydrochloride (Lederle), terramycin hydrochloride (C. Phizer & Co.), and tetracycline hydrochloride (also from C. Phizer & Co.). The sulphanilamide, sulphamezathine, terramycin, and tetracycline were pure; commercial samples of the other antibacterials were used.

(c) *Statistical Analysis*

The results have been subjected to analysis of variance. Where a number of independent treatments have been compared with controls, the standard error of the difference between a treatment and the control mean has been calculated using the formula (Cochran and Cox 1950):

$$sd = \sqrt{\{s^2[(1/r_1) + (1/r_2)]\}},$$

where  $sd$  = standard error of the difference between treatment and control means,

$s^2$  = error mean square from the analysis of variance,

$r_1$  = number of control replications, and

$r_2$  = number of treatment replications.

The significance of difference between the means has then been assessed by the  $t$ -test using  $sd$  and the degrees of freedom associated with  $s^2$ .

TABLE 1

MEAN MOTILITY INDICES OVER A 6-HR PERIOD FOR SIX FOWL EJACULATES IN DILUENTS CONTAINING ANTIBACTERIALS

Mean of five controls for each ejaculate was 77.1 and asterisks are used to denote a significant fall in motility

Antibacterial	Concentrations (mg/ml)					
	0.02	0.1	0.5	0.2	1	5
Aureomycin	77.7	81.3	71.0	—	—	—
Terramycin	77.8	76.3	56.5**	—	—	—
Tetracyn	77.3	77.5	76.3	—	—	—
Penicillin	—	—	—	74.3	64.5**	45.3**
Streptomycin	—	—	—	77.2	75.2	73.0
Sulphanilamide	—	—	—	72.2	53.8**	20.2**
Sulphamezathine	—	—	—	72.5	66.5*	73.5
Chloromycetin	—	—	—	65.2**	26.3**	0.5**

\* $P < 0.05$ .      \*\* $P < 0.01$ .

## III. RESULTS

(a) *Preliminary Motility Observations*

The effects of varying concentrations of eight antibacterials on six fowl ejaculates over a 6-hr period are shown in Table 1. From the analysis of variance in which each level of each antibacterial was considered as an independent treatment the standard error of the difference between treatment and control means was found to be 4.2 (i.e.  $\sqrt{[89 \times (6/30)]}$ ), and has been used for the  $t$ -test (degrees of freedom = 135). Penicillin and sulphanilamide depressed the motility of fowl spermatozoa at 1 mg/ml and chloromycetin was toxic even at 0.2 mg/ml. The effects of penicillin at 1 mg/ml and chloromycetin at 0.2 mg/ml, however, were

not very marked and in other tests these concentrations did not always cause a significant depression of motility. It may be noted that ejaculates often varied considerably in their susceptibility to the antibacterials. The other antibacterials were well tolerated and, except for terramycin at 0.5 mg/ml, had no significant effect on motility during the 6-hr period, even at the highest concentrations used, which were near the limits of solubility.

TABLE 2  
COMPARISON OF THE TOXICITY OF ANTIBACTERIALS IN DILUENTS OF  
RELATIVE TONICITY 100 AND 140

Results are the mean motility indices for four ejaculates over 4 hr.  
0.9 per cent. NaCl = 100 per cent. tonicity

Tonicity	Antibacterial	Concentration (mg/ml)			
		0	0.2	1	5
100	Penicillin	45	44	35	16
	Sulphanilamide	45	36	17	0
	Streptomycin	42	47	39	32
140	Penicillin	56	56	53	36
	Sulphanilamide	57	55	44	9
	Streptomycin	57	58	57	56

When another series of tests was performed using a similar diluent containing less glucose to give a relative tonicity of 100 (i.e. of optimum tonicity for mammalian spermatozoa), the antibacterials usually depressed motility more severely. In some instances, particularly with streptomycin, sulphanilamide, and sulphamezathine the spermatozoa adapted themselves to the antibacterial and the fourth-hour score was higher than the first-hour score. Using the fourth-hour score as unit observation it was found that, in addition to the antibacterials that were toxic in the diluent of higher tonicity, sulphamezathine (1 mg/ml), terramycin and tetracycline (0.1 mg/ml), and aureomycin (0.5 mg/ml) also decreased motility.

A comparison of the toxicity of penicillin, sulphanilamide, and streptomycin in the two diluents is shown in Table 2. Motility indices were significantly lower in diluents of tonicity 100 and the antibiotics were more toxic than at tonicity 140 (i.e. there was a significant tonicity  $\times$  levels of antibiotic interaction).

#### (b) *Penicillin and Streptomycin in Combination*

Since penicillin and streptomycin are used together in diluents for bull semen, these antibacterials in combination were added to the diluent for fowl semen at a concentration of 1 mg/ml. The combination was not significantly toxic in these tests.

(c) *Effects of Antagonists*

There is evidence that the antibacterial action of sulphanilamide, chloromycetin, and aureomycin may be antagonized by *p*-aminobenzoic acid, phenylalanine, and biotin respectively (Woods 1940; Woolley 1950; Osteux and Laturage 1952).

TABLE 3

EFFECT OF SOME ANTIBIOTICS AND THEIR ANTAGONISTS ON THE MEAN MOTILITY INDEX OF FOWL SPERMATOOA

Number of replications shown in parentheses. Analysis of variance for the effects of aureomycin on biotin toxicity is also given

Substance	Concn. (mg/ml)	Motility Index	Substance	Concn. (mg/ml)	Motility Index	Substance	Concn. (mg/ml)	Motility Index
Control	—	51 (5)	Control	—	49 (5)	Control	—	55 (4)
Sulphanilamide	1	25 (5)	Chloro- mycetin	0.5	2 (5)	Aureo- mycin	0.2	52 (4)
<i>p</i> -Aminobenzoic acid	0.2	50 (5)	Phenyl- alanine	0.5	46 (5)	Biotin	2	27 (4)
Sulphanilamide + <i>p</i> -amino- benzoic acid	1 0.2	48 (5)	Chloro- mycetin + phenyl- alanine	0.5 0.5	3 (5)	Aureo- mycin + biotin	0.2 2	48 (4)

## Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio
Effect of biotin	1	69.8**
Effect of aureomycin	1	23.6*
Ejaculate differences	3	3.6
Interactions		
Biotin × aureomycin	1	40.5**
Biotin × ejaculates	3	0.6
Aureomycin × ejaculates	3	0.2
Residual	3	14

\* $P < 0.05$ .    \*\* $P < 0.01$ .

The spermicidal properties of the first two antibacterials, however, were unaffected by concentrations of *p*-aminobenzoic acid (0.2 mg/ml) and phenylalanine (0.5 mg/ml) innocuous to fowl spermatozoa (Table 3). *p*-Aminobenzoic acid proved spermicidal in higher concentrations than 1 mg/ml and similar concentrations of phenylalanine also significantly depressed motility. Aureomycin as might be



TABLE 4  
EFFECT OF DILUTION ON THE TOXICITY OF ANTIBACTERIALS FOR FOWL SPERMATOOZA  
Each motility index is the mean of four ejaculates over a 4-hr period

Penicillin			Sulphanilamide			Chloromycetin			Terramycin		
Concn. (mg/ml)	Sperm Dilution		Concn. (mg/ml)	Sperm Dilution		Concn. (mg/ml)	Sperm Dilution		Concn. (mg/ml)	Sperm Dilution	
	1 in 3	1 in 20		1 in 3	1 in 20		1 in 3	1 in 20		1 in 3	1 in 20
0	58	53	0	59	54	0	56	51	0	54	51
5	49	31	1	57	29	0.5	53	9	0.5	53	15
10	38	20	2	54	21	1	36	2	1	53	11
20	36	10	4	45	13	2	20	2	2	52	7

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio			
		Penicillin	Sulphanilamide	Chloromycetin	Terramycin
Dilution effect	1	85.7**	160.0**	200.1**	1384.0**
Antibiotic concentration	3	62.4**	35.2**	105.8**	152.2**
Ejaculate differences	3	4.0*	10.3**	8.5**	25.8**
Interactions					
Dilution × antibiotic	3	5.5*	11.3**	23.7**	126.2**
Dilution × ejaculate	3	1.5	1.7	7.8**	11.3**
Antibiotic × ejaculate	9	0.6	0.3	0.7	1.1
Residual	9	26	30	36	6

\*  $P < 0.05$ .\*\*  $P < 0.01$ .

expected from the initial experiments was not spermicidal; biotin, however, was toxic at a concentration of 2 mg/ml and aureomycin (0.2 mg/ml) was found to antagonize this effect (Table 3).

(d) *Effect of Seminal Plasma on the Toxicity of Antibacterials*

The effects of penicillin, sulphanilamide, chloromycetin, and terramycin on the motility of fowl semen diluted 1 in 3 and 1 in 20 in modified Ringer's solution, with summary analyses of variance, are shown in Table 4. In all cases, the antibacterials were more toxic when the semen was diluted 1 in 20.

TABLE 5  
EFFECT OF 30 PER CENT. SEMINAL PLASMA ON THE TOXICITY OF ANTIBACTERIALS  
TO FOWL SPERMATOOA  
Results are the mean motility indices over 4 hr for four ejaculates diluted 1 in 20

Antibiotic	Concn. (mg/ml)	Modified Ringer	30% Plasma in Modified Ringer
Control	—	46	54
Penicillin	20	11	38
Sulphanilamide	4	17	54
Chloromycetin	0.5	29	57
Terramycin	2	21	51

The increased susceptibility of fowl spermatozoa at low cell concentrations is clearly due to the dilution of seminal plasma since the toxicity of the antibiotics was reduced at a 1 in 20 dilution, if 30 per cent. seminal plasma in modified Ringer's solution was used (Table 5).

(e) *Metabolic Studies*

Table 6 shows the effect of spermicidal concentrations of penicillin, sulphanilamide, and chloromycetin on the oxygen uptake, fructolysis, and lactic acid production of fowl spermatozoa. Analyses of variance gave residual mean squares of 0.55 (oxygen uptake), 40.0 (fructose utilization), and 68.0 (lactic acid production)—all with 9 degrees of freedom—and these have been used to calculate the standard error for the *t*-test.

(i) *Penicillin*.—In general, metabolism was little affected. There was, however, a statistically significant depression of oxygen uptake ( $t = 2.2$ ,  $P = 0.05$ ) and anaerobic fructolysis ( $t = 6.2$ ,  $P < 0.01$ ).

(ii) *Sulphanilamide*.—Oxygen uptake, aerobic and anaerobic fructolysis, and lactic acid production were all consistently depressed. In the presence of sulphanilamide, as in the controls, fructose utilization and lactic acid production was greater under anaerobic than under aerobic conditions.

(iii) *Chloromycetin*.—Chloromycetin almost completely inhibited oxygen uptake. Under aerobic conditions, fructose breakdown and lactic acid production in the presence of chloromycetin was as good as, if not better than, the controls. Under anaerobic conditions, however, there was no increase in fructose utilization or lactic acid production as occurred in the controls.

TABLE 6

EFFECT OF HIGH CONCENTRATIONS OF ANTIBACTERIALS ON THE METABOLISM OF FOWL SPERMATOOZOA OVER 3 HR AT 25°C

Mean values for four replications are given and asterisks are used to denote significant differences from control values

Antibiotic	Concn. (mg/ml)	Oxygen Uptake ( $\mu$ l/10 <sup>8</sup> cells)	Fructose Utilization ( $\mu$ g/10 <sup>8</sup> cells)		Lactic Acid Production ( $\mu$ g/10 <sup>8</sup> cells)	
			Aerobic	Anaerobic	Aerobic	Anaerobic
Control	—	7.6	56	92	44	84
Penicillin	20	6.5*	47	64**	41	71
Sulphanilamide	5	4.3**	12**	47**	10**	53**
Chloromycetin	5	1.8**	62	51**	59*	55**

\* $P < 0.05$ .      \*\* $P < 0.01$ .

At the end of each experiment the motility of the spermatozoa in the Warburg flasks and anaerobic tubes was checked. Motility was most depressed by chloromycetin; the effects of sulphanilamide and penicillin were less striking.

#### IV. DISCUSSION

The most important point that emerges from these studies is the relative toxicity of penicillin and sulphanilamide to fowl spermatozoa. Both antibacterials are routinely added to bull semen in artificial insemination practice and are well tolerated by mammalian spermatozoa in general. Thus under similar conditions White (1954) found that sulphanilamide and penicillin were usually non-toxic to mammalian spermatozoa in concentrations of up to 5 mg/ml. Sulphathiazole is apparently toxic to fowl spermatozoa (Smith 1949) but sulphamezathine is less harmful.

Fowl spermatozoa are also rather more susceptible to chloromycetin. A concentration of at least 1 mg/ml was needed to depress the motility of mammalian spermatozoa (White 1954) whereas 0.2 mg/ml proved toxic in the experiments reported here. As might be expected from their close structural relationship the spermicidal activity of aureomycin, terramycin, and tetracycline is very similar and, in general, does not differ much for fowl and mammalian spermatozoa.

Fowl spermatozoa also resemble the other spermatozoa studied in their tolerance to streptomycin which was innocuous even at the highest dose tried. This would seem a desirable antibacterial for use in the artificial insemination of poultry.

Wilcox and Shorb (1958) state that a combination of terramycin and streptomycin at low concentration gives better fertility than streptomycin and penicillin in combination, although both are efficient in controlling bacterial growth in fowl semen.

Ejaculates may vary in their susceptibility to antibacterials and, furthermore, the degree of dilution of the semen is an important factor. The results presented here clearly indicate that seminal plasma counters the spermicidal activity of a number of antibiotics. The mechanism is, however, not very specific and it seems unlikely that such structurally diverse antibacterials could all be inactivated by seminal plasma. On the other hand, the seminal plasma may help maintain the integrity of the cell surface; in its absence there is probably a general increase in permeability and a more rapid passage of antibiotics into spermatozoa.

The finding that two members of the vitamin B complex, viz. biotin and *p*-aminobenzoic acid, are toxic to spermatozoa is surprising; the spermicidal concentrations are, however, vastly in excess of those encountered in the tissues. Phenylalanine has previously been reported toxic to bull spermatozoa on shaking in air and its action is probably dependent on the formation of hydrogen peroxide by the enzyme L-amino acid oxidase (Tosic and Walton 1950).

The antagonism of the spermicidal effects of biotin by aureomycin is of interest in view of the report by Osteux and Laturage (1952) that biotin counters the action of aureomycin on enzyme preparations from *Clostridium welchii*. Both observations suggest that aureomycin and biotin might form a biologically inactive complex so that the properties of either may be masked by the presence of the other.

Because of the limited sensitivity of the methods, the metabolic experiments had to be done on semen diluted only 1 in 3 and, even at very high concentrations, the antibacterials did not have marked spermicidal effects. Nevertheless, it has been possible to demonstrate a significant depression of oxygen uptake by penicillin, sulphanilamide, and chloromycetin and a decreased ability of the spermatozoa to break fructose down to lactic acid. The action of chloromycetin is particularly interesting in that it inhibits the marked Pasteur effect shown by fowl spermatozoa, i.e. the increase in fructose breakdown and lactic acid production under anaerobic conditions. With ram and bull spermatozoa high concentrations of chloromycetin inhibit both aerobic and anaerobic glycolysis almost completely (White 1954).



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# THE GLYCOLYSIS AND REDUCING ACTIVITY OF RAM SPERMATOOA IN PHOSPHATE-CONTAINING MEDIA

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## *Summary*

The reduction of 2,3,5-triphenyltetrazolium chloride by ram spermatozoa was linear in the range of cell densities from  $1.0 \times 10^8$  to  $8.0 \times 10^8$ /ml.

Tetrazolium was reduced more rapidly under anaerobic conditions than aerobically unless fructose was present, when reduction was quite rapid in air.

Phosphate stimulates the reduction of tetrazolium, the breakdown of fructose, and the accumulation of lactic acid.

Both phosphate and succinate stimulate the reduction of tetrazolium by washed ram spermatozoa.

## I. INTRODUCTION

The metabolism of spermatozoa is greatly influenced by the conditions imposed in their study. Thus Bishop and Salisbury (1955) have shown that phosphate ions depress the respiration of unwashed bull spermatozoa at body temperature in comparison with that occurring in 0.9 per cent. sodium chloride. Similar results have been obtained using washed ram and bull spermatozoa with fructose and glycerol as substrates (Mann and White 1956, 1957; White 1957). The reduction in respiration is accompanied by an accumulation of lactic acid (Salisbury and Nakabayashi 1957).

The reducing activity of bull semen has been studied by Smith, Mayer, and Merilan (1956, 1957*a*, 1957*b*) who used a manometric method to measure succinic dehydrogenase activity. Mohri (1957), in studies of sea-urchin spermatozoa, measured the reduction of 2,3,5-triphenyltetrazolium chloride by the succinic dehydrogenase system.

Tetrazolium salts have the property of acting as hydrogen receptors and are reduced by certain dehydrogenases to insoluble formazan pigments. Although widely applied for the histochemical localization of enzymes in many tissues, tetrazolium salts have been little used in studies of spermatozoa (Mohri 1957; Blackshaw 1958; King and Mann 1959).

The following experiments describe the use of 2,3,5-triphenyltetrazolium chloride to demonstrate the overall metabolic activity of ram spermatozoa particularly in the presence of phosphate ions.

## II. MATERIALS AND METHODS

Ram semen was collected by the electrical production of ejaculation (Blackshaw 1954); only ejaculates of good initial motility were used.

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The diluents employed were of varying constitution but for the preliminary tests the medium of White (1953) was found suitable. This contains 0.04M NaCl, 0.048M  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ , 0.032M  $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$ , and 200 mg per cent. fructose; in some tests the fructose was omitted. Otherwise the diluents were prepared from 0.154M NaCl to which was added isotonic phosphate buffer or 0.15M sodium succinate. The phosphate buffer contained 71 volumes of 0.13M  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$  and 29 volumes of 0.17M  $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$  per 100 ml to give pH 7.0. The osmotic pressure of the media in some tests was varied to give relative tonicity values of 125, 100, and 75 where the tonicity of 0.154M NaCl is 100. These various levels were prepared by dilution of a 125 tonicity solution with water. Where necessary 200 mg per cent. fructose was added.

In most experiments, the incubation of the semen was carried out under anaerobic conditions in an atmosphere of nitrogen. The dilution rate of the semen was 1 in 5 and washed suspensions of spermatozoa were prepared by diluting semen 1 in 10 with 0.154M NaCl and centrifuging for 10 min at 1500 r.p.m. (approximately 300 *g*) for 10 min. The supernatant liquid was removed and 0.154M NaCl added to restore the volume. Two further centrifugations were performed with removal of the supernatants and after the last treatment the volume was adjusted to that of the original semen. In the tests of washed semen, the control samples were centrifuged under the same conditions and the packed cells resuspended each time.

Lactic acid was estimated by the method of Barker and Summerson (1941) and fructose by the method of Mann (1948). The reducing activity of the spermatozoa was determined by the reduction of 2,3,5-triphenyltetrazolium chloride to the insoluble red formazan. A suitable concentration of the tetrazolium salt was found to be 0.25 mg/ml and at the end of the incubation period 1 ml of the diluted semen was mixed with 6 ml of acetone. The formazan was readily extracted and the optical density of the coloured solution obtained was measured at 485  $m\mu$ . A series of standard solutions of 2,3,5-triphenyltetrazolium chloride were prepared containing 0.05, 0.04, 0.03, 0.02, and 0.01 mg/ml. Excess sodium hydrosulphite was added and the resulting insoluble formazan extracted with acetone. The absorption spectra of the formazan produced by the reducing activity of spermatozoa was found to be identical to that produced by the sodium hydrosulphite.

In all cases the accumulation of lactic acid, the disappearance of fructose, and the reduction of tetrazolium were measured in  $\mu\text{g}/10^8$  cells/hr. The results were examined statistically by the analysis of variance.

### III. RESULTS

An initial experiment was performed to examine the effect of cell density on the reduction of tetrazolium. Semen was washed three times to remove the seminal plasma and resuspended in the isotonic buffer of White (1953) containing 200 mg per cent. fructose. Cell densities of  $10^8$ ,  $2 \times 10^8$ ,  $4 \times 10^8$ , and  $8 \times 10^8$  per ml were used, and the reduction of tetrazolium determined after incubation for 2 hr at 37°C.

The analysis of variance of the results for six ejaculates showed a highly significant effect for linear regression ( $F_{(1,15)} = 57.1$ ,  $P < 0.01$ ). The variance ratios for the quadratic and cubic effects were  $F_{(1,15)} = 1.4$  and 0 respectively,  $P > 0.05$ . A regression line was calculated,  $E = 1.2 + 27.6X$ , where  $E$  is the amount ( $\mu\text{g}$ ) of tetrazolium reduced and  $X$  is  $\log_2$  (sperm number). The sperm number is taken as 1, 2, 4, or 8, the total sperm count being the product of the sperm number with  $10^8$ . It is clear that over the range of spermatozoal densities likely to occur in the usual metabolic tests the cell density does not influence the rate of reduction of tetrazolium.

TABLE 1

EFFECTS OF WASHING, NITROGEN, AND FRUCTOSE ON THE ACCUMULATION OF LACTIC ACID AND THE REDUCTION OF TRIPHENYLTETRAZOLIUM CHLORIDE BY RAM SPERMATOZOA (MEANS OF FOUR REPLICATIONS)

Treatment	Gas Phase	Fructose (mg %)	Lactic Acid ( $\mu\text{g}/10^8$ cells/hr)	Triphenyl-tetrazolium Chloride ( $\mu\text{g}/10^8$ cells/hr)
Unwashed	Air	0	69.2	8.5
	Air	200	235.5	9.7
	Nitrogen	0	214.2	12.2
	Nitrogen	200	208.2	13.3
Washed	Air	0	1.3	6.2
	Air	200	208.2	11.7
	Nitrogen	0	1.3	12.7
	Nitrogen	200	162.5	9.7

The effect of incubation under anaerobic conditions was studied along with the effects of washing the spermatozoa free of seminal plasma and the addition of 200 mg per cent. fructose. The spermatozoal suspensions were incubated in Thunberg tubes in air or they were evacuated with a water pump and flushed with nitrogen. A factorial plan was used and, because of the limited amount of semen available, a  $2^3$  design with the three-factor interaction confounded with blocks was found suitable. Four replications were performed using individual ejaculates from eight rams. A summary of results for the reduction of tetrazolium and the accumulation of lactic acid is given in Table 1.

Analysis showed that anaerobic conditions favour the reduction of tetrazolium by ram spermatozoa particularly in the absence of fructose. On the other hand, the removal of seminal plasma by washing significantly reduced the accumulation of lactic acid, the effect being particularly marked under anaerobic conditions. Overall, anaerobic conditions stimulated the accumulation of lactic acid in the absence of added fructose but reduced it when fructose was present. Although the advantage of anaerobic conditions was not apparent for the reduction of tetrazolium in the presence of fructose, further tests were conducted under nitrogen as fructose was not included in all diluents.



Phosphate media have marked effects on the metabolism of bull semen and studies were made to determine the occurrence of similar possible effects with ram semen. Sodium chloride, a phosphate buffer, and a mixture of equal volumes of each were compared at relative tonicities of 75, 100, and 125 (0.154M NaCl = 100).

TABLE 2

EFFECTS OF TONICITY AND THE PRESENCE OF PHOSPHATE ON THE GLYCOLYSIS AND THE REDUCTION OF TRIPHENYLTETRAZOLIUM CHLORIDE BY RAM SPERMATOZOA (MEANS OF FOUR REPLICATIONS)

Relative Tonicity (0.9% NaCl = 100)	Sodium Chloride	Phosphate	Lactic Acid ( $\mu\text{g}/10^8$ cells/hr)	Fructose ( $\mu\text{g}/10^8$ cells/hr)	Triphenyl-tetrazolium Chloride ( $\mu\text{g}/10^8$ cells/hr)
125	+	—	62.2	7.0	3.1
	+	+	79.5	91.2	19.9
	—	+	121.2	93.2	37.4
100	+	—	28.5	23.8	4.9
	+	+	184.0	133.8	24.6
	—	+	149.0	102.0	29.9
75	+	—	25.8	28.0	8.5
	+	+	169.0	79.0	21.1
	—	+	149.2	142.0	27.8

Fructose (200 mg per cent.) was included in all the media. Summary results for the reduction of tetrazolium, the accumulation of lactic acid, and the disappearance of fructose are given in Table 2. Phosphate greatly stimulated fructolysis and the

TABLE 3

EFFECT OF PHOSPHATE ON THE GLYCOLYSIS AND REDUCING ACTIVITY OF RAM SPERMATOZOA (MEANS OF SIX EJACULATES)

Phosphate (M)	Lactic Acid ( $\mu\text{g}/10^8$ cells/hr)	Fructose ( $\mu\text{g}/10^8$ cells/hr)	Triphenyl-tetrazolium Chloride ( $\mu\text{g}/10^8$ cells/hr)
0.0000	48.7	31.5	3.0
0.0015	51.0	39.1	2.6
0.0060	61.3	40.6	3.9
0.0240	142.7	97.6	8.2
0.0960	142.7	106.6	11.3

reduction of tetrazolium but, although part replacement of chloride increased the accumulation of lactic acid, complete replacement had no further effect.

The next experiment also examined replacement of chloride by phosphate but over a phosphate range of 0.0015M, 0.006M, 0.024M, and 0.096M. Similar effects

were obtained (Table 3) and analysis showed highly significant linear effects for lactic acid, fructose, and tetrazolium.

The reduction of tetrazolium has been used to demonstrate the activity of succinic dehydrogenase in various tissues, and in the following tests the effects of phosphate and succinate were studied on washed and unwashed spermatozoa. The semen was washed three times in 0.154M NaCl and then suspended in saline containing 0.02M sodium phosphates, 0.02M sodium succinate, or the same levels of both substances. The reduction of tetrazolium after incubation for 3 hr at 37°C was estimated and the mean values for eight ejaculates are given in Table 4. It is

TABLE 4  
EFFECTS OF PHOSPHATE AND SUCCINATE ON THE REDUCTION  
OF TRIPHENYLTETRAZOLIUM CHLORIDE BY RAM SPERMATOOZA  
(MEANS OF EIGHT REPLICATIONS)

Diluent	Triphenyltetrazolium Chloride Reduced ( $\mu\text{g}/10^8$ cells/hr)	
	Control	Washed
Chloride	3.7	2.3
Phosphate	12.2	10.4
Succinate	10.0	10.0
Phosphate + succinate	14.4	9.4

clear that both phosphate and succinate markedly stimulated reduction of tetrazolium by unwashed cells and that a combination of the two is even better. However, after washing, although both substances stimulated reduction, a combination of the two did not further stimulate it.

#### IV. DISCUSSION

The reduction of some tetrazolium salts by the activity of cells or isolated enzyme systems is influenced by the gaseous phase present (Nachlas *et al.* 1957) and triphenyltetrazolium chloride has been shown by Hershey, Cruickshank, and Mullins (1958) to require relatively anaerobic conditions for reduction by skin in media containing succinate and phosphate. However, in the presence of added fructose ram spermatozoa appear to reduce tetrazolium to only a slightly less extent in air than anaerobically.

An early report by Lardy and Phillips (1943) indicated that phosphate is required for the maintenance of glycolysis by spermatozoa but has an inhibitory effect on respiration. Recent observations by Bishop and Salisbury (1955) showed that the motility and respiration of bull spermatozoa are severely inhibited by phosphate. Also Salisbury and Nakabayashi (1957) showed that the utilization of

fructose and the accumulation of lactic acid by bull spermatozoa were increased by phosphate. This increase in lactic acid appears to be partly due to the inhibitory effect of phosphate on the oxidation of the acid by spermatozoa (Mann and White 1957; White 1957). However, it is clear that phosphate greatly stimulates the reduction of tetrazolium by ram spermatozoa and also increases the rate of fructolysis.

The reduction of tetrazolium by washed spermatozoa is stimulated by succinate as well as phosphate. Both these ions have been shown by Kearney, Singer, and Zastrow (1955) and Kearney (1957) to combine with the active centre of succinic dehydrogenase and enhance its activity. It is well known that inorganic phosphate plays an essential role in the Embden-Meyerhof glycolytic scheme; in normal brain it is necessary for the oxidation of pyruvate (Mellwain, Buchel, and Cheshire 1951) and for the formation of pyruvate from fumarate (Long 1945). On the other hand, inorganic phosphate inhibits glucose 6-phosphate dehydrogenase, the enzyme catalyzing the first reaction of the hexose monophosphate shunt (Theorell 1935; Kravitz and Guarino 1958). A high phosphate level could inhibit the shunt and allow glycolysis to proceed whereas a low level could exert opposite effects. However, Salisbury and Nakabayashi (1957) have not been able to demonstrate the shunt in bull spermatozoa suspended in chloride media.

#### V. ACKNOWLEDGMENTS

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# A STUDY OF THE REPRODUCTIVE TRACT OF *HELIX ASPERSA* MÜLLER AFTER PARTIAL GONADECTOMY

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## Summary

Surgical gonadectomy was attempted in *H. aspersa*; control operations were also carried out. Mortality was high among both groups of operated animals.

The attempts at gonadectomy proved ineffective in that the surviving animals were found at dissection to have viable remnants of gonadal tissue located in pockets among the tissues of the digestive gland. It is concluded that surgical gonadectomy in this genus is not practicable since such remnants persist for at least 3 months after operation.

A total of 10 animals (6 gonadectomies, 4 controls) survived operation by periods of 7–13 weeks. The albumen glands and common ducts of these animals were subjected to histological study. No significant difference could be detected between the tissues of the gonadectomized animals and those of the controls. It is concluded that partial gonadectomy does not affect the tissues in question, and it is suggested that the genital tract of the Helicidae might not be under hormonal control of the gonad.

## I. INTRODUCTION

An annual reproductive cycle has been reported in several gastropods: *Arion rufus* and *Helix aspersa* (Filhol 1938a, 1938b); *Limax maximus* (Abeloos 1943); *H. pomatia* (Ancel 1903; Baecker 1932); *Physa fontinalis* (Duncan 1958). According to Filhol (1938a) spermatogenesis ceases in winter in most pulmonates. Baecker (1932) has recorded cytological changes in the albumen gland of *H. pomatia* during the egg-laying season. Filhol (1938a) noted a decrease in size of the albumen gland of *H. aspersa* in winter. The same worker (Filhol 1938b) described cytological changes in the tissues of the common duct of *H. aspersa* which could be correlated with onset of winter: the epithelial cells lining the lumen of the oviducal portion of the common duct become charged with lipid, and the mucous cells outside this epithelium show regressive changes; the prostatic tissue located along the spermiducal portion of the common duct also shows regressive changes. Filhol interpreted these findings as indicating a cessation of functional activity during the winter months.

There is some evidence for gonadal control of the genital tract in gastropods. Abeloos (1943) succeeded in carrying out castration on an unspecified number of specimens of *L. maximus*. He reported that at dissection the oviducts of castrates showed no signs of glandular swelling and the albumen glands were small ("... l'oviducte ne montre aucun gonflement glandulaire et la glande de l'albumine est rudimentaire."), a condition contrasting with that seen in controls dissected at the same time of year (November). Abeloos suggested that the genital tract of

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*Limax* is under hormonal control of the gonad. Laviolette (1950) succeeded in carrying out grafting experiments on several species of the Arionidae and the Limacidae. He reported that grafts of gonad tissue taken from adult specimens of *Mesarion subfuscus* caused macroscopic changes in the genital tracts of two immature hosts of *A. rufus* within 1 month of implantation. The same worker reported that a fragment of ovispermiduct from a young specimen of *A. rufus* was implanted in the body cavity of an adult *Kobeltia hortensis* and was recovered 5 weeks later; the fragment had increased in size and showed marked histological differentiation (details not given) towards the adult condition. Laviolette stated that similar results were obtained with homografts of albumen gland material in which two specimens of *L. flavus* were involved. He also succeeded in carrying out gonadectomy on immature specimens of *A. rufus* and on an unspecified number of mature individuals of *L. maximus*. Three months after operation the latter were dissected. Macroscopic examination revealed a diminution in size of the genital tracts and particularly in the size of the albumen glands. The latter findings (except with regard to the albumen gland) do not agree with those of Abeloos mentioned above. Abeloos clearly states that 4 months after operation neither the penis nor that portion of the hermaphrodite duct left *in situ* shows any sign of change, and the oviduct merely fails to show the increase in size noted in the controls.

Studies have been carried out on the genital tracts of certain gastropods suffering from parasitic castration. This work has been reviewed by Hanström (1939). In some genera (*Paludina*, *Limnaea*, *Planorbis*) no effect has been observed, but in *Littorina* regressive changes have been described, and these changes have been taken to indicate gonadal control of the reproductive tract. However, the effects of parasitic castration must be interpreted with care, and such evidence cannot be regarded as conclusive.

The present study was undertaken to discover whether gonadectomy in *Helix* was practicable with a view to ascertaining the effect of that operation in a genus in which it has not yet been attempted.

## II. METHOD

The snails used in this study were at least  $2\frac{1}{4}$  cm in diameter, measured across the widest portion of the shell (at right angles to the longitudinal axis of the animal). In each animal the apex of the shell was removed with fine forceps so as to lay bare an area of the hump approximately 1 cm in diameter (Fig. 1). The apical whorl of the hump was then opened out centrifugally so as to reveal the greyish-coloured gonad. The integument was broken along the length of this region (parallel with the direction of curvature) and stripped away on either side, so as to lay bare the gonad.

Gonadal tissue was removed with fine forceps until the darker tissue of the digestive gland was revealed. Care was taken to avoid damaging the latter tissue. A careful search was made for the hermaphrodite duct at the base of the gonad; this duct was severed.

At conclusion of the operation the hump was replaced in position and the shell closed with "Elastoplast". Failure to do this led to high mortality, apparently

due to desiccation. In the case of control operations, the same procedure was followed but no gonadal tissue was removed and the hermaphrodite ducts were not severed. At dissection the gonadal region of the hump, the albumen gland, and the common duct of each animal were fixed in Zenker, mounted in paraffin wax, and sectioned. The stains used were Harris's haematoxylin and eosin.

### III. RESULTS

Of a large number of snails operated on between the months of September and November, 10 (i.e. 4 controls, 6 gonadectomies) survived\* for periods of 7–13 weeks. They were dissected (with one exception) in January. The results given



Fig. 1.—*H. aspersa*, view from above showing portion of shell removed in operation and position of the gonad relative to the surrounding tissues of the visceral hump:

1, ovotestis; 2, albumen gland; 3, digestive gland.

below are derived from these animals, all of which were apparently in good condition at dissection. Removal of the "Elastoplast" revealed that in all cases the shells had been repaired by a whitish, calcareous deposit, which effectively closed the holes in the shells but did not resemble the original shell material either in colour or in texture. The remainder of the operated animals died within 7 weeks of operation; the deaths were apparently due to infection of the wounds.

#### (a) Effectiveness of Operational Procedure

The effectiveness of the method used was ascertained by histological examination of the gonad region. It was discovered that in no case was gonadectomy complete. It had seemed that severe damage to the ovotestis at operation might have resulted in rapid degeneration of any gonadal tissue left *in situ*; this did not prove to be the case. There was evidence of regressive changes in the gonadal

\* The term "survived" is used here for convenience. It does not imply that the animals were dissected *post mortem*; they were clearly capable of surviving the operation by longer periods than those given above.



remnants of only one animal (S.8), dissected 13 weeks after operation (Plate 1, Fig. 2). In this animal some of the "follicles" left *in situ* were empty of all germinal elements except occasional oocytes, and some of the latter were evidently in process of being resorbed. The follicles contained very large granular cells with light-staining nuclei. Many of these cells showed large cytoplasmic vacuoles containing a yellowish granular material. Although their lineage is uncertain, it is at least possible that they are hypertrophied "yolk cells" (cf. Gatenby 1917) and there seems little doubt that they were responsible for resorption of the germinal elements. There is clear evidence that in some of the follicles the cells in question were being replaced by connective tissue.

There is no reason to think that the follicles described above had been damaged at the time of operation: their boundaries were intact and some were located on the periphery of the gonad, well away from the locus of operation. Apart from these follicles, the animal (S.8) possessed a number of intact gonadal remnants which contained spermatozoa and oocytes (Plate 1, Fig. 1). All the other gonadectomies (including another specimen dissected 13 weeks after operation) possessed similar remnants containing spermatozoa and oocytes and showing no evidence of regression. It therefore appears that remnants of the ovotestis of *Helix* begin to undergo regression some 3 months after partial gonadectomy, and that up to this time such remnants must be regarded as viable.

On the other hand, there was no evidence of regeneration of damaged or excised genital tissue. Lavolette (1954) reported that castrated specimens of *A. rufus* commenced regeneration of the excised gonads within 3 to 6 weeks after operation. However, Abeloos (1943) found no such regeneration in his specimens of *L. maximus*. It appears that the capacity for regenerating lost gonadal tissue is not general among pulmonates.

#### (b) Histology

(i) *The Albumen Gland.*—The histology of the albumen gland of *Helix* has been described: Baecker (1932), Filhol (1938a), Yung (1911). The gland is acinar, surrounded by a thin layer of connective tissue. In small glands the acini are round or oval in cross section, but in large glands they tend to be polygonal and closely packed. Each acinus consists of a layer of secretory epithelium, one cell thick, surrounding a central lumen. The acini are demarcated by a delicate investment of connective tissue. The glandular cells are markedly eosinophil, with basal nuclei. In small glands the cells tend to be low columnar, with relatively large, rounded nuclei (Plate 2, Fig. 3); the cytoplasm has the general appearance of a reticulum, and in some of the cells it is possible to distinguish vacuoles containing an eosinophil colloidal material.

In large glands the acini are large and the lumina are occluded (Plate 2, Fig. 4). The cell boundaries are difficult to distinguish, but when seen they show the cells to be large and polygonal, occupying the whole acinus. The nuclei are small and pyknotic, and are located against the peripheral connective tissue. The cytoplasm is filled with large globules of eosinophil colloidal material. In some cells the globules have apparently fused, forming a mass of colloid which occupies most



of the cell. In such cases the colloidal mass is surrounded by a thin layer of cytoplasm, and broken cytoplasmic threads are visible within the mass. Such glands are dense and friable and tend to be difficult to embed. The fixative caused shrinkage cracks to appear, sometimes around individual cells and sometimes around the colloidal globules. Baecker (1932) found a similar difficulty with the albumen gland of *H. pomatia* and recommended that use of fixatives containing potassium bichromate be avoided—a precaution considered unnecessary in the present work.

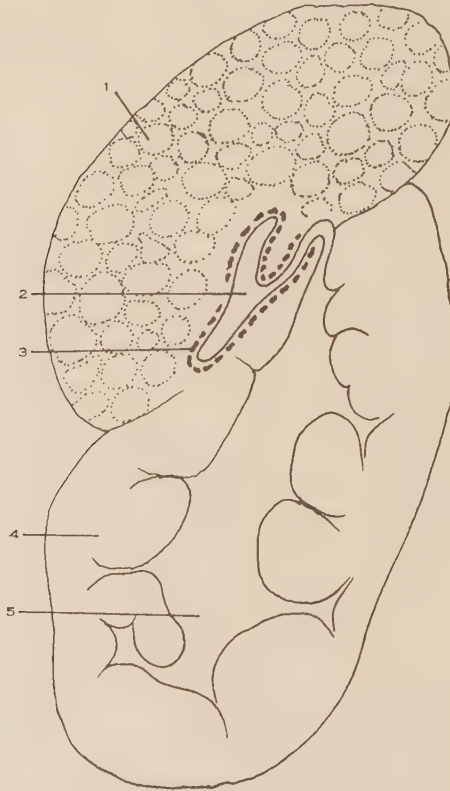


Fig. 2.—Diagrammatic cross section of common duct of *H. aspersa*: 1, acini of prostate; 2, spermiduct; 3, layer of basophil cells surrounding spermiduct; 4, mucous epithelium of oviduct; 5, oviduct.

The controls used in the present investigation showed both of the pictures described above (cf. Plate 2, Figs. 3 and 4). Other snails (not operated on) dissected at the same time of year revealed all stages in the process of transformation from the one "type" of albumen gland to the other. It is impossible to say at this stage whether the two types of gland represent differences in physiological activity (and therefore recur in regular annual sequence) or whether the former type is characteristic of young snails and the latter of older animals. The former type is certainly characteristic of young snails, but it appears that both types may occur in older snails. This observation supports the view that the two histological types signify

differences in physiological activity rather than differences in age. This is borne out by the work of Yung (1911) and by that of Baecker (1932), both of whom report a cessation of secretory activity of the albumen gland of *H. pomatia* during the winter months.

The albumen glands of two of the gonadectomized animals are shown in Plate 3, Figures 5 and 6. Despite the evident differences between Plate 2, Figures 3 and 4 (controls), on the one hand, and Plate 3, Figures 5 and 6, on the other, there is considered to be no consistent histological difference between the albumen glands of the gonadectomized animals and those of the controls. It is therefore concluded that partial gonadectomy causes no histological change in the albumen gland of this species, at least not within 3 months of the operation.

(ii) *The Common Duct*.—The histology of the common duct of *Helix* has also been described: Yung (1911), Baecker (1932), Filhol (1938b). In cross section the spermiducal and oviducal portions\* are clearly distinguished (Fig. 2). The oviduct is of large diameter with folded walls. The lumen is bounded by a ciliated epithelium outside of which is a high columnar epithelium which probably secretes the mucous envelopes for the eggs. The cells of the latter epithelium are faintly basophil and are sometimes vacuolated. They may be relatively small, with well-marked boundaries and rounded nuclei, or relatively large, with obscure boundaries and pyknotic nuclei. In the latter case the thickness of the epithelium is increased in proportion to the increase in size of the individual cells. Thus, the appearance of the mucous epithelium surrounding the oviduct varies considerably from animal to animal, apparently depending on the physiological state of the tissue. The mucous epithelium is bounded externally by a connective tissue sheath.

The spermiduct is of relatively small internal diameter. The lumen invariably lies at an angle to that of the oviduct from which it is partially separated by two folds or ridges of tissue. According to Filhol (1938b) these folds of tissue, developed in the region of the slit connecting the two lumina, can ensure almost complete separation of the two ducts.

The lumen of the spermiduct is lined with a ciliated epithelium continuous with that of the oviduct. Outside the ciliated epithelium there is a layer of markedly basophil cells, usually one cell thick (Plate 4, Fig. 7). The basophil cells are irregularly ovoid, with coarsely reticular cytoplasm and an eccentric or peripheral nucleus which it is usually difficult to detect. Each cell has a delicate cytoplasmic prolongation which passes between the ciliated cells of the surface epithelium and reaches the lumen of the spermiduct (Plate 5, Fig. 9). Similar cells were found in *H. pomatia* by Baecker (1932) who described and figured the cytoplasmic prolongations extending toward the lumen of the duct. The layer of basophil cells may completely surround the spermiduct, but more frequently is more or less restricted to one side of it. Outside the basophil cells is located the "prostatic tissue".

The prostate consists of numerous acini separated by delicate strands of connective tissue. Each acinus usually has a small central lumen surrounded by a

\* Henceforward these two subdivisions of the common duct will be referred to as the "spermiduct" and "oviduct" respectively. Note that the vas deferens and oviduct *sensu stricto* are not included in this study.

columnar epithelium of eosinophil cells. The latter have rounded, basal nuclei and a coarsely granular cytoplasm. The prostatic tissue is bounded externally by a connective tissue sheath which is continuous with that of the oviduct and therefore forms an investment for the common duct.

There appears to be no significant histological difference between the common ducts of the gonadectomized animals and those of the controls except in the following respect: the layer of basophil cells surrounding the spermiduct was found to be absent from two controls (cf. Plate 4, Figs. 7 and 8). The significance of this observation is not clear. It is probably not related to the experimental procedure.

It is therefore concluded that partial gonadectomy causes no histological changes in the tissues of the common duct of *H. aspersa*, at least not within 3 months of the operation.

#### IV. DISCUSSION

##### (a) *Albumen Gland*

The function of this gland in gastropod molluscs is known. In 1938 Baldwin and Bell showed that the albumen gland of *H. pomatia* secretes a complex galactose polymer which was referred to as "galactogen" since it produces galactose on hydrolysis. Duncan (1958), working on the albumen gland of *Physa fontinalis*, obtained similar results and also carried out a number of tests for other substances: "mucous, proteins, mucopolysaccharides and other sugars". The results of these tests were negative. Duncan cited the work of two other investigators in support of the view that galactogen is secreted by the albumen glands of many gastropods and is not peculiar to pulmonates.

In *Physa* each ovum is given an envelope of galactogen by the albumen gland before being invested with a double membrane and enclosed within an egg capsule. The galactogen constitutes the food supply of the embryo (Duncan 1958), and there is little doubt that it serves a similar purpose in *Helix*.

##### (b) *Common Duct*

It seems to be generally agreed that the glandular cells surrounding the oviduct in pulmonates are mucous-secreting and provide envelopes for the ova (Filhol 1938b; Duncan 1958). Duncan has produced experimental evidence that in *P. fontinalis* the glandular tissue of the oviduct (here separate from the vas deferens) secretes two membranes around each ovum as well as the capsular material in which a number of these "eggs" are enclosed. Details of the process of egg formation in *Helix* are not available, but it is no doubt similar to that in *Physa*, with the oviducal epithelium contributing investments for the ova.

The prostatic tissue of *Helix* is clearly defined. There is little doubt that it is analogous to, and homologous with, the prostate of *Physa* which Duncan (1958) has described as consisting of blind, finger-like follicles opening into the vas deferens. Yung (1911) has described the prostate of *H. pomatia*, the main elements of which are glandular, prismatic cells with reticular cytoplasm containing numerous calcareous granules ("granulations calcaires"). Yung put forward the view that these cells provide the eggs with the calcareous envelopes with which they are surrounded,

but this postulate was later questioned by Baecker (1932). Yung's suggestion hardly takes account of the anatomical relationships of the prostate which so evidently forms part of the male duct system.

Duncan (1958) has produced evidence that the prostate of *Physa* produces alkaline phosphatase and possibly amino acids. He took the view that the secretions of the prostate provide a fluid medium for transfer of the spermatozoa at copulation. It may be tentatively concluded that in *Helix* the prostate has a similar function.

The basophil cells which surround the spermiduct are mucous-secreting (Baecker 1932). It seems possible that they are analogous to, if not homologous with, the cells of the praeputial gland of *Physa*. Duncan (1958) has described the latter as consisting of basophil cells with long cytoplasmic necks passing through a layer of muscle tissue and reaching to the lumen of the praeputium (a sac-like structure, distal to the penis sheath, through which the penis is everted at copulation). According to Duncan the cells of the praeputial gland "produce mucins which presumably facilitate the eversion of the penis".

A praeputial gland does not occur in *Helix*. However, the basophil cells of *Helix* are strongly reminiscent of the cells of the praeputial gland of *Physa* in their structure (notably the long necks reaching to the lumen of the male duct), their mucous-secreting function, and their association with the male duct system. The fact that they occur along the spermiduct suggests that the secretion they produce is useful as a constituent of the seminal fluid and is not primarily a penial lubricant.

### (c) Conclusion

As already mentioned, there is some evidence that the gonad exerts hormonal control over the glandular tissues of the reproductive tract in the Arionidae and the Limacidae (Abeloos 1943; Laviolette 1950). A similar situation is known in the vertebrates, where the accessory sex organs are controlled by hormones produced by the testes and ovaries. However, the results given here at least suggest caution in assuming that the same applies in the Helicidae. There is no doubt that most of the gonadal tissue was removed from the gonadectomized snails on which this study is based, and it was reasonable to expect signs of histological regression in any tissues controlled by the gonads. There was no evidence of histological regression in the albumen glands or in the glandular tissues of the common ducts, and the conclusion suggested is that these tissues are not controlled by the gonad.

However, the evidence presented is not conclusive, since it can be argued that the gonadal remnants were capable of maintaining the tissues in question in normal physiological (and histological) condition. Clearly a more effective method of gonadectomy is indicated—a method capable of complete removal or destruction of the gonadal tissue. In vertebrates the use of X-rays for the latter purpose is common practice, and the method might well be feasible in the case of *Helix*.

### V. ACKNOWLEDGMENT

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## EXPLANATION OF PLATES 1-5

## PLATE 1

- Fig. 1.—Animal S.8, dissected 13 weeks after partial gonadectomy. Intact gonadal remnant, showing follicles containing spermatozoa and oocytes.  $\times 75$ .  
 Fig. 2.—Animal S.8 showing degeneration of a gonadal remnant. A number of the follicles contain oocytes, but spermatozoa are absent. Note very large cells occupying follicles. Arrows indicate connective tissue which is evidently in process of replacing the large cells occupying the follicles.  $\times 75$ .

## PLATE 2

- Fig. 3.—Portion of albumen gland of a control. In this animal the gland was small. Some of the acini are seen in longitudinal section.  $\times 150$ .  
 Fig. 4.—Portion of albumen gland of a control. In this case the gland was large. Note relatively large acini occupied by cells containing large globules of colloidal material. Fixation has caused shrinkage cracks which delimit the colloidal globules as well as individual cells.  $\times 150$ .

## PLATE 3

- Fig. 5.—Portion of albumen gland of an animal dissected 11 weeks after partial gonadectomy. Acini intermediate in size between those of the controls shown in Plate 2, Figures 3 and 4. Cells show marked vacuolation.  $\times 150$ .  
 Fig. 6.—Animal S.8, dissected 13 weeks after partial gonadectomy. Portion of albumen gland. Acini smaller than in control shown in Plate 2, Figure 4, but histology is similar.  $\times 150$

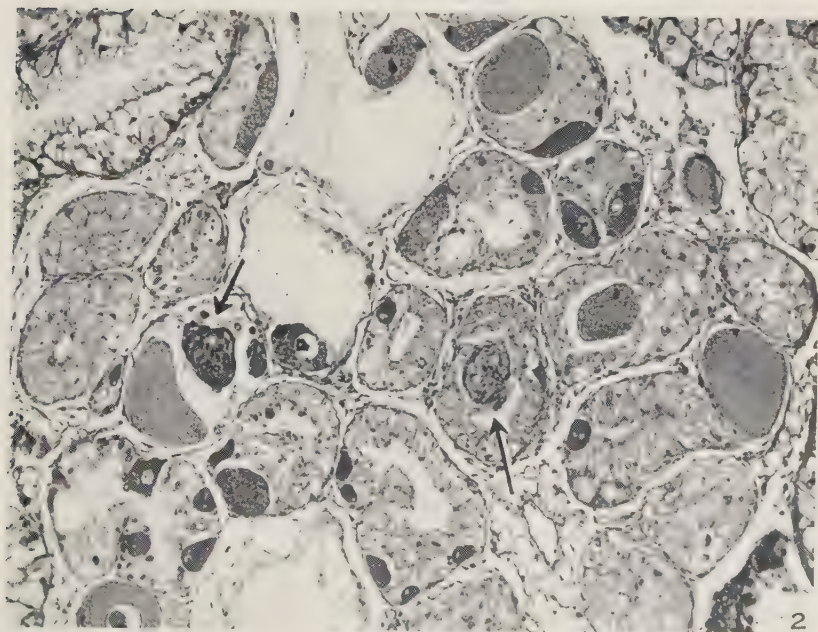
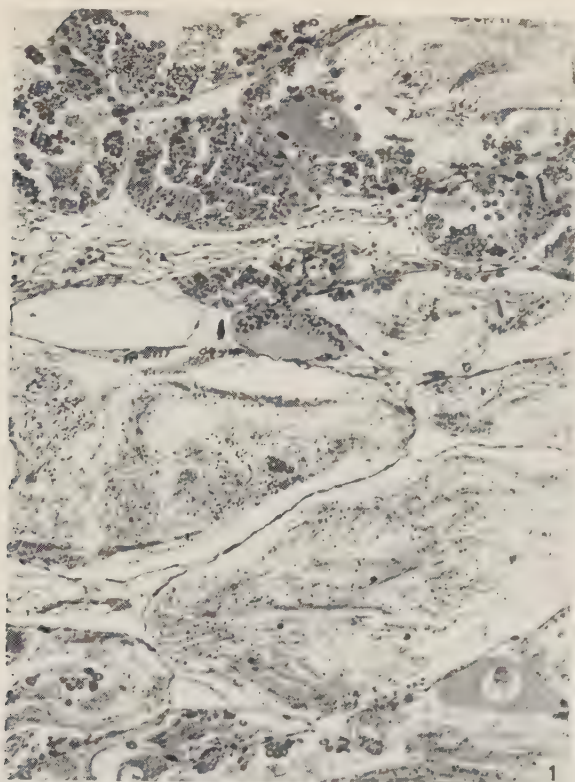
## PLATE 4

- Fig. 7.—Cross section of common duct of a control in region of spermiduct. Arrows indicate the layer of basophil cells surrounding the spermiduct (*s*). Prostatic tissue occupies the upper portion of the field and mucous epithelium of the oviduct occupies the lower portion.  $\times 150$ .  
 Fig. 8.—Cross section of common duct of a control; similar field to that shown in Plate 4, Figure 7. Note absence of basophil cells from region surrounding spermiduct (*s*).  $\times 150$ .

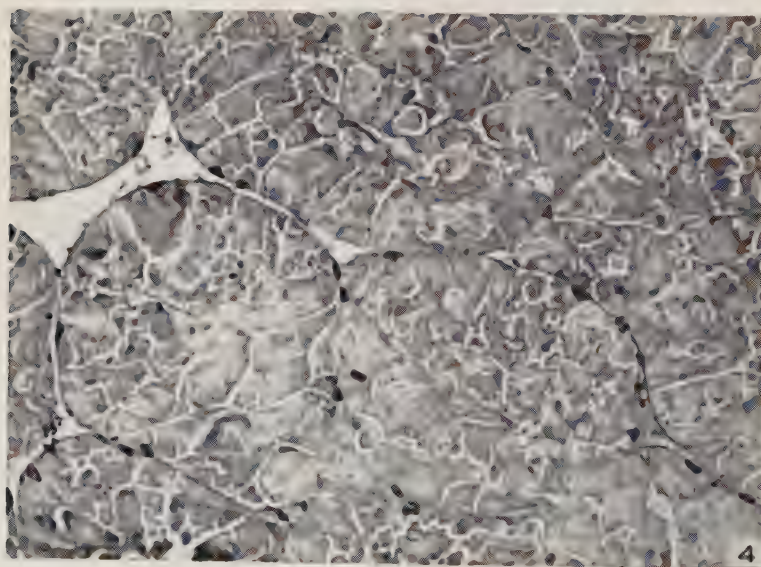
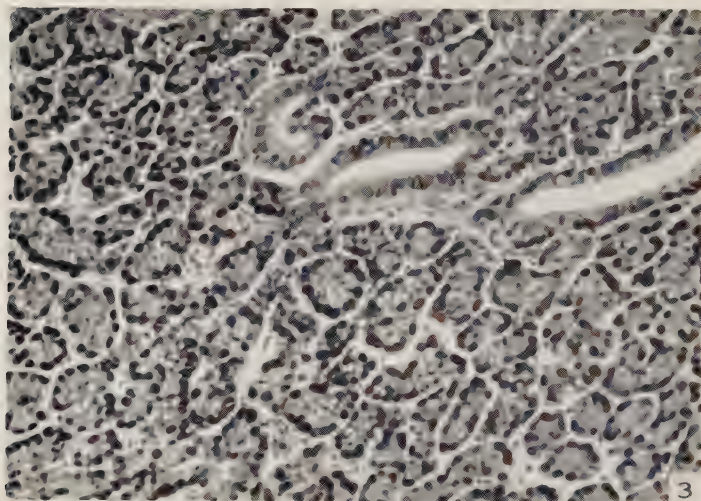
## PLATE 5

- Fig. 9.—Cross section of common duct of animal S.8, dissected 13 weeks after partial gonadectomy. Similar field to that shown in Plate 4, Figures 7 and 8. Note layer of basophil cells surrounding spermiduct (*s*). Arrow indicates neck of a basophil cell passing between ciliated epithelial cells of spermiduct to reach the lumen.  $\times 150$ .

REPRODUCTIVE TRACT OF HELIX ASPERSA

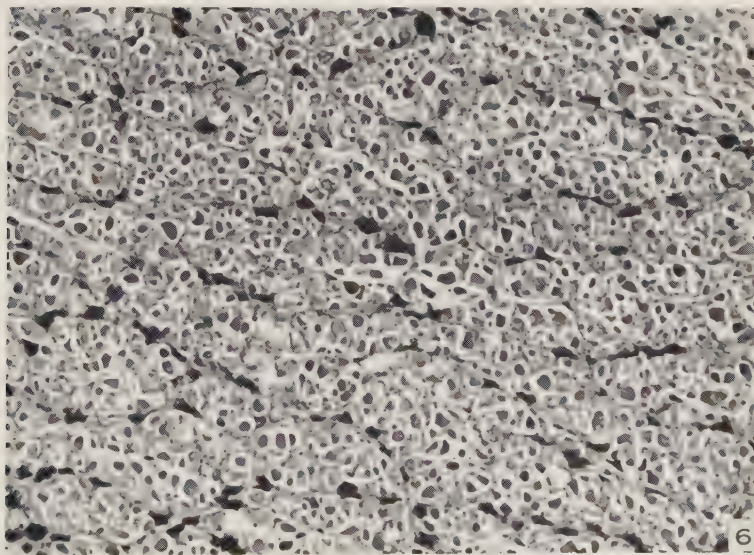
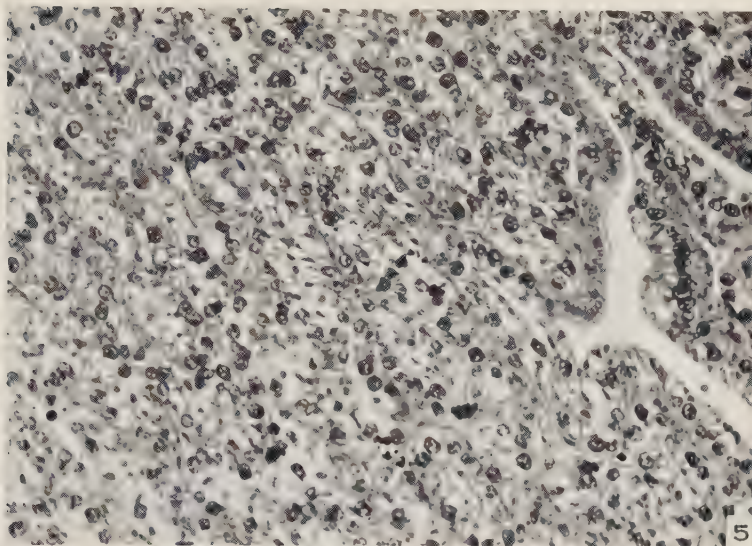


REPRODUCTIVE TRACT OF HELIX ASPERSA



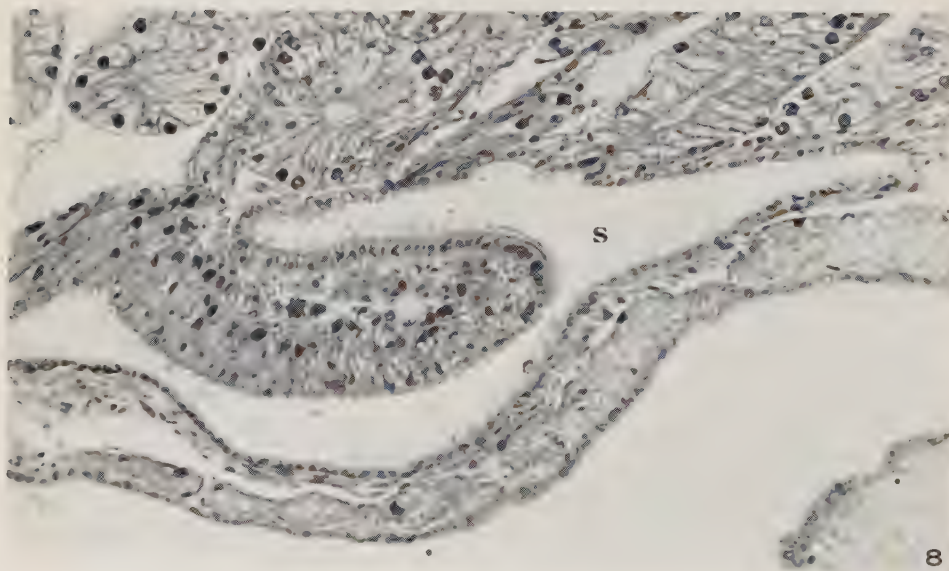
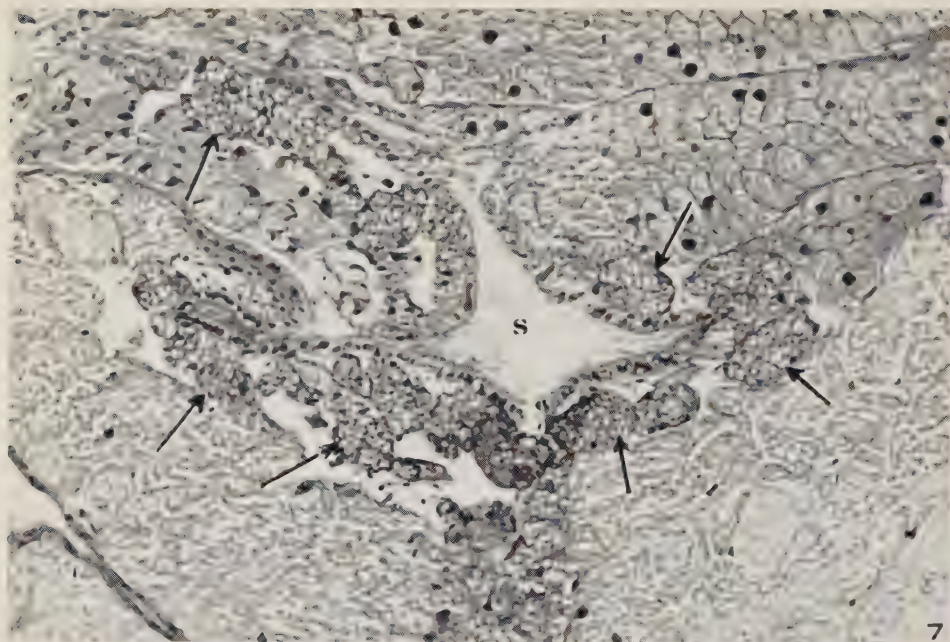


REPRODUCTIVE TRACT OF *HELIX ASPERSA*

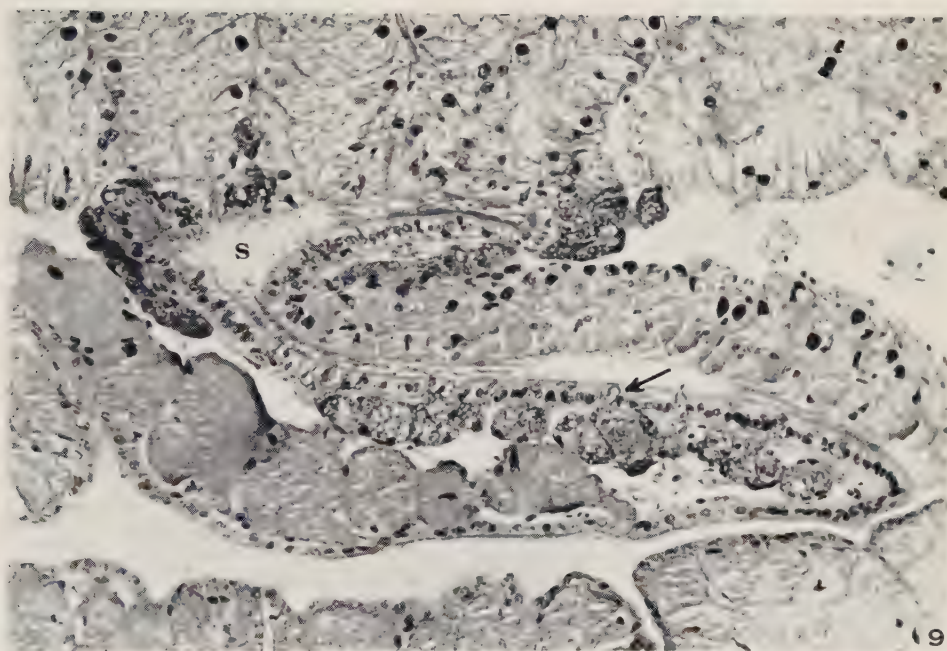




REPRODUCTIVE TRACT OF HELIX ASPERSA



REPRODUCTIVE TRACT OF HELIX ASPERSA





# THE OXIDATION OF CYSTEINE TO SULPHATE IN SOIL

By J. R. FRENEY\*

[Manuscript received February 22, 1960]

## *Summary*

The oxidation of cysteine to sulphate by a mixed population of soil micro-organisms was studied in a perfusion unit. Intermediates in this reaction were identified by paper chromatography and by an enrichment-reperfusion technique.

The results obtained by these two methods suggest that cysteinesulphinic acid, cysteic acid, sulphite, and  $\beta$ -hydroxypyruvic acid were involved in this oxidation. Sulphide, which has often been postulated as an intermediate in the conversion of organic sulphur to sulphate, could not be detected in the system nor in the gases emanating from the system.

## I. INTRODUCTION

Much of the work performed on the decomposition of organic sulphur compounds which may occur in soil has been effected with pure cultures of micro-organisms (see Frederick, Starkey, and Segal 1957). These species may be unimportant members of the vast community of soil organisms. Also it is possible that the kinetics and pathway of sulphophication in soil are completely different from those relating to pure cultures. While there can be no question of the great importance of studies with pure cultures, it is obvious that if we wish to know which reactions are important in soil then it is necessary to perform the study with the soil itself.

For these reasons an investigation was conducted into the oxidation of cysteine to sulphate in soil contained in a perfusion unit. An earlier report (Freney 1958) showed that cystine and cystine disulphoxide were involved in this transformation. This paper reports the results of experiments designed to identify further intermediates in this reaction.

## II. EXPERIMENTAL

The transformation of cysteine to sulphate in soil was studied in a perfusion unit of the type described by Lees and Quastel (1944). The principle of this technique has been adequately described by these two authors (1944, 1946).

In the system under investigation, 400 ml of 0.01M cysteine hydrochloride solution was perfused through 30 g of air-dried soil crumbs (2-5 mm fraction). The solution was cycled at such a rate that waterlogging of the soil did not take place. The soil used in most of these studies was a reddish brown clay loam derived from basalt.

Amino acids in the perfusate were detected, and a preliminary identification made, by two-dimensional paper chromatography. Details of the methods used were recorded in a previous paper (Freney, Delwiche, and Johnson 1959). In

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addition, amino acids containing sulphur were detected by an iodine-azide reagent (Block, Durrum, and Zweig 1958). Larger amounts of amino acids were isolated by chromatography on Whatman No. 3 paper (Block, Durrum, and Zweig 1958) in *n*-butanol-acetic acid-water (4 : 1 : 5 v/v) and *tert.*-butanol-water (7 : 3 v/v).

Keto acids in the perfusate were isolated as the 2,4-dinitrophenylhydrazones and identified by conversion to their respective amino acids by catalytic hydrogenation (Towers, Thompson, and Steward 1954; Meister and Abendschein 1956).

TABLE 1  
 $R_F$  VALUES FOR CYSTEINESULPHINIC AND CYSTEIC ACIDS IN A NUMBER OF  
CHROMATOGRAPHIC SYSTEMS

Amino Acid	Whatman Paper No.	Solvent (v/v)	$R_F$
Cysteinesulphinic acid	4	<i>n</i> -Butanol-acetic acid-water (4 : 1 : 5)	0.16
	4	<i>tert.</i> -Butanol-water (7 : 3)	0.33
	1	Phenol-water (4 : 1)	0.13
	3	<i>n</i> -Butanol-acetic acid-water (4 : 1 : 5)	0.11
Cystaic acid	4	<i>n</i> -Butanol-acetic acid-water (4 : 1 : 5)	0.10
	1	Lutidine-collidine-water (1 : 1 : 1)	0.46
	1	Methanol-water-pyridine (20 : 5 : 1)	0.44
	1	<i>n</i> -Butanol-pyridine-water (1 : 1 : 1)	0.26

The enrichment-reperfusion technique (Gleen and Quastel 1953) was used to provide further evidence that certain compounds were intermediates in this transformation. Briefly, this technique involves perfusing a solution of cysteine hydrochloride through soil until sulphate production reaches a maximum. Sulphate is then removed from the perfusion system by washing with water. As a result of this treatment the soil has a population of microorganisms and a system of enzymes capable of immediately oxidizing to sulphate, cysteine, or any of the intermediates in its transformation to sulphate, i.e. without a lag phase. (Soil thus treated will be referred to as the enriched soil.) If the substance reperused after cysteine is not an intermediate in this transformation then a lag phase would occur while systems are built up which are capable of oxidizing the new substrate to sulphate.

Sulphate was determined by Johnson and Nishita's reduction-methylene blue method (1952). The soil in the perfusion unit was analysed for sulphide by digesting the soil with aluminium metal and hydrochloric acid. Any hydrogen sulphide evolved was determined by the methylene blue method. The gases emanating from the perfusion system were passed through a trap containing zinc acetate, and the contents of the trap were analysed for sulphide plus sulphate by Johnson and Nishita's method. The zinc nitroprusside reaction described by Feigl (1947) was used to detect sulphite.

The results obtained with the soil described above were confirmed with other soils. The soils used in this study were:

- (1) Dublin soil, California, U.S.A.
- (2) Reddish chocolate soil derived from basalt, Walcha, N.S.W.
- (3) Lateritic podzolic soil derived from orstein and granite, Uralla, N.S.W.
- (4) Krasnozom derived from basalt, Lismore, N.S.W.
- (5) Red-brown earth derived from sediments, Griffith, N.S.W.
- (6) Solodic soil derived from sediments, Ashford, N.S.W.
- (7) Lateritic podzolic soil derived from granite, Beerwah, Qld.
- (8) Chocolate soil derived from basalt, Merriwa, N.S.W.

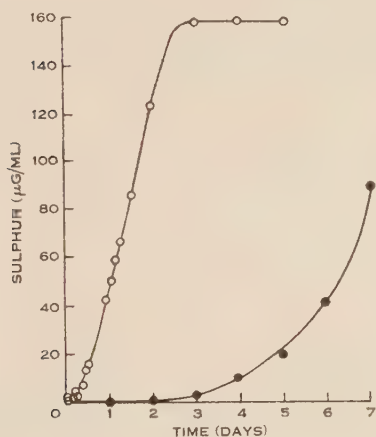


Fig. 1

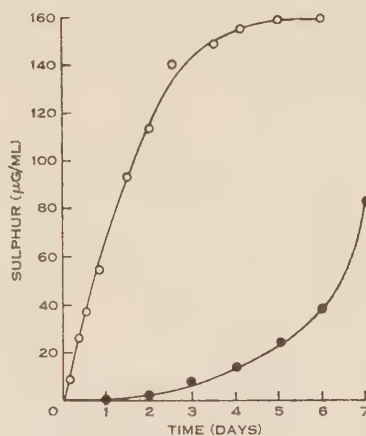


Fig. 2

Fig. 1.—Sulphate production from a 0.01N cysteinesulphinic acid solution perfused through an enriched soil (○) and a normal soil (●).

Fig. 2.—Sulphate production from a 0.01N cysteic acid solution (pH adjusted to 7) perfused through an enriched soil (○) and a normal soil (●).

The results obtained in the perfusion unit were compared with those obtained in a soil *in situ*. A cysteine hydrochloride solution was applied to a soil in the field, and the area covered with a plastic sheet to prevent leaching by rain. After a period of 48 hr, the area was sampled and the samples extracted with normal hydrochloric acid. Amino acids in the evaporated extract were detected by paper chromatography.

The sulphur-containing amino acids were obtained from the California Foundation for Biochemical Research, Los Angeles, U.S.A., and other amino acids from British Drug Houses Pty. Ltd., Poole, England.

### III. RESULTS

Chromatograms of the perfusate revealed the presence of a number of amino acids. One of these amino acids and cysteinesulphinic acid ran as a single spot when chromatographed together under a variety of conditions. Another amino acid appeared to be cysteic acid when tested by a similar procedure. The  $R_F$  values found for these amino acids under these conditions are given in Table 1.

When 0.01N solutions of cysteinesulphinic acid and cysteic acid (adjusted to pH 7 with sodium hydroxide) were reperfused through enriched soils, sulphate was produced immediately. However, when these solutions were perfused through untreated soil, sulphate was produced only after a lag period of 2 days (see Figs. 1 and 2).

In addition to the two amino acids containing sulphur, three other amino acids (serine, alanine, and glutamic acid) were identified in the perfusate by co-chromatography. The corresponding keto acids ( $\beta$ -hydroxypyruvic acid, pyruvic acid, and  $\alpha$ -ketoglutaric acid) were detected by chromatography of, and catalytic hydrogenation of, their 2,4-dinitrophenylhydrazones.

Sulphite was detected in the perfusate by the zinc nitroprusside reaction. It was not possible to prove that sulphite and sulphide were intermediates by reperfusing these substances through enriched soils because of their autoxidation to sulphate.

Sulphide could not be detected in the soil, the solution, or in the gases emanating from the system.

The same spectrum of amino acids was found with all the soils studied in the perfusion units, and for the soil *in situ*.

#### IV. DISCUSSION

The identification of cysteinesulphinic acid and cysteic acid in the perfusate by paper chromatography, and the immediate oxidation of these two compounds when perfused through enriched soils strongly suggests that they were intermediates in the transformation of cysteine to sulphate.

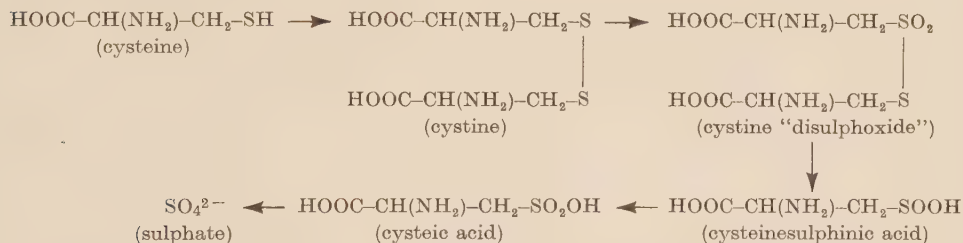
It is logical to assume that, in the perfusing system, cysteic acid was formed directly from cysteinesulphinic acid by oxidation. Kearney and Singer (1953a, 1953b) showed that this oxidation can occur in cell-free extracts of *Proteus vulgaris*, but they inferred that only a small amount of cysteinesulphinic acid was converted to sulphate via cysteic acid. Singer and Kearney (1955) concluded that the main pathway for the catabolism of cysteinesulphinic acid was via a transamination reaction with either  $\alpha$ -ketoglutaric acid or oxaloacetic acid to form  $\beta$ -sulphonylpyruvic acid,  $\text{HOOC-CO-CH}_2\text{-SO}_3\text{H}$ . Desulphination of this substituted pyruvic acid would then yield pyruvic acid and sulphite; the latter being oxidized to sulphate. They also concluded that cysteic acid was catabolized through the medium of  $\beta$ -sulphonylpyruvic acid,  $\text{HOOC-CO-CH}_2\text{-SO}_3\text{H}$ .

However, neither  $\beta$ -sulphinyl- nor  $\beta$ -sulphonylpyruvic acids could be detected in the perfusate by chromatography of the 2,4-dinitrophenylhydrazones, nor could they be detected as cysteinesulphinic acid or cysteic acid, respectively, when the mixed hydrazones were converted to the parent amino acids by catalytic hydrogenation. It is possible that these two substituted pyruvic acids are so transitory that derivatives could not be formed, and thus their presence could not be detected. Therefore, the possibility that cysteinesulphinic acid and cysteic acid were catabolized via these substituted pyruvic acids could not be eliminated.

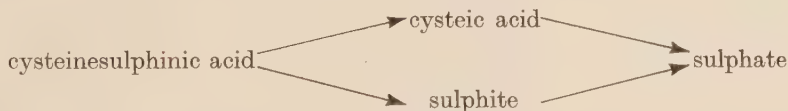
The presence of serine and its analagous keto acid,  $\beta$ -hydroxypyruvic acid, suggest that they are involved in the transformation under study. It seems unreasonable to expect that  $\beta$ -hydroxypyruvic acid should be formed in the system by side reactions when other keto acids (apart from the key metabolites pyruvic and  $\alpha$ -ketoglutaric acids) were not detected. Desulphuration reactions yielding alanine described by Singer and Kearney (1955) would not explain the presence of serine and  $\beta$ -hydroxypyruvic acid in this system. The mechanism by which cysteine-sulphinic acid and cysteic acids decompose to yield sulphite or sulphate, serine, and  $\beta$ -hydroxypyruvic is unknown.

That sulphide could not be detected in the system does not prove unequivocally that it was not involved in the transformation under study.

The results presented above, together with those obtained previously (Freney 1958), suggest that the *most probable* pathway for the oxidation of cysteine to sulphate in soil is:



It is possible that the overall reaction may involve a number of side or branched reactions, for example:



and that the whole process may be due to a number of different microorganisms.

Evidence to support the suggestion that cysteinesulphinic acid was formed directly from cystine "disulphoxide" was reported by Sweetman (1959), who found that this reaction occurred *in vitro*.

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# THE CHROMATOGRAPHY OF INSULIN ON DEAE-CELLULOSE IN BUFFERS CONTAINING 8M UREA\*

By E. O. P. THOMPSON† and I. J. O'DONNELL†

[*Manuscript received March 2, 1960*]

## *Summary*

The chromatography of four samples of insulin by elution analysis at constant pH and ionic strength on a diethylaminoethyl (DEAE)-cellulose column has been studied. The presence of three components was apparent at pH 7.4 in a buffer containing 8M urea. The results are compared with those obtained on the same samples of insulin by other workers using countercurrent and chromatographic techniques. The experimental and theoretical curves for the major peak of the International standard insulin sample coincide.

## I. INTRODUCTION

In a previous paper (O'Donnell and Thompson 1960) it was shown that when various insulin preparations were chromatographed on diethylaminoethyl (DEAE)-cellulose in the pH range 7-9 they could be separated into a major and a minor protein component. It was not found possible under a variety of conditions of pH, ionic strength, and temperature to separate from the main peak the desamido insulin, varying amounts of which exist in most insulin preparations (Harfenist and Craig 1952). To enable chromatography to be carried out at lower pH values near the isoelectric point, where insulin is normally insoluble, and under disaggregating conditions, buffers containing 8M urea have been used. Under these conditions four samples of insulin have each been separated into at least three components. Comparison has been made with countercurrent separations on the same insulins (Harfenist and Craig 1952; Human and Leach 1960).

Cole (1959) has given a preliminary account of the separation of the commercial insulin preparations into three components by chromatography on an acidic resin, "Amberlite IRC-50", with buffers containing 8M urea.

## II. EXPERIMENTAL

### *(a) Columns*

The preparation and operation of the DEAE-cellulose columns were as described in a previous paper (O'Donnell and Thompson 1960). The DEAE-cellulose was equilibrated with buffer containing 8M urea and packed under 10 lb/sq.in. nitrogen pressure in a 13-17 by 0.9 cm (dia.) column kept at constant temperature. The column was then further equilibrated overnight by flowing buffer through it until the influent and effluent pH values were identical. The column was loaded with 0.5 ml of a 2 per cent. solution of insulin, which was washed in with filtered

\* A preliminary account of this work was presented to the Australian Biochemical Society at Canberra in January 1960.

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buffer ( $2 \times 0.5$  ml) and then eluted at a flow rate of 12–20 ml/sq.cm/hr with buffer which had passed through a subsidiary 5 cm column of DEAE-cellulose. This smaller column was connected by a ground-glass joint to the top of the main column to filter the urea buffer. This filter, which was designed to remove ultraviolet-absorbing material and dark impurity, also collected carbonate ions. If, after use, the filter was washed with 1N potassium chloride, coloured bands were eluted together with material absorbing strongly at 276  $m\mu$ .

Approximately 0.7 ml fractions of the eluate were collected by a drop-counting fraction collector and the exact size of the fraction determined by weighing. The density of the buffers was 1.11.

A detergent for minimizing drop-size variation was not added to the buffers and the fraction size (12 drops) showed a marked decrease on emergence of protein (see Fig. 2).

#### *(b) Buffer and Insulin Solutions*

The buffer used contained 8M urea (B.D.H. "Analar"), 0.01M Tris (tris-(hydroxymethyl)aminomethane), and 0.001M "Versene" and it was adjusted to the required pH with hydrochloric acid. The insulin spontaneously dissolved in the buffer and this solution was adjusted to the correct pH with 1N Tris solution.

The insulin samples chromatographed were International sample No. 2189 (Anon 1957), Boots sample No. 9011G (Boots Pure Drug Co., Nottingham, England), Lilly sample No. 535664 (Eli Lilly and Co., Indianapolis, U.S.A.), and a sample from the Commonwealth Serum Laboratories (C.S.L.), Parkville, Vic.

#### *(c) Analysis of Effluent Fractions*

The effluent fractions were diluted with 3 ml water and if necessary the pH adjusted to about 7.5 with a drop of hydrochloric acid of suitable normality. The absorption of light was measured at 276  $m\mu$  in 1 cm cells using a Beckmann DU spectrophotometer. Following the procedure of Goodwin and Morton (1946) the absorption was also measured at 320 and 360  $m\mu$  and corrections for scattering made to the 276  $m\mu$  value. No activities of the separated insulin components were determined.

#### *(d) Characteristics of the Column*

The method of Mayer and Tompkins (1947) was used to calculate the theoretical elution curve using the parameters obtained with insulin on the DEAE-cellulose column. For this purpose it was necessary to know the hold-up volume (volume of mobile liquid held between the swollen cellulose particles) of the column. This was determined by a method based on that of Pepper, Reichenberg, and Hale (1952); a small column, 5 cm long and fitted with a sintered disk as described by these authors, was packed in buffer with DEAE-cellulose under the same conditions as those used for the larger column. After allowing the liquid to drain to the level of the cellulose the tube plus cellulose was placed in a graduated centrifuge tube, centrifuged at 400  $g$  for 20 min and the volume of emerging liquid measured. Centrifugation at 500  $g$  did not increase this volume which was close to two-thirds of the total volume of the packed column. The centrifuged pellet was washed free

of buffer on a Buchner funnel, dried at 100°C, and weighed. This gave a value of 6.2 ml (5.9, 6.6, 6.0) as the hold-up volume per gram of dry DEAE-cellulose for a column operated under these conditions. Thus our columns had a hold-up volume of 8–9 effluent fractions, depending on the length of the column, and this agreed with the tube number in which the fraction size suddenly decreased (see Fig. 2) and often with the emergence of a small amount of front-running impurity.

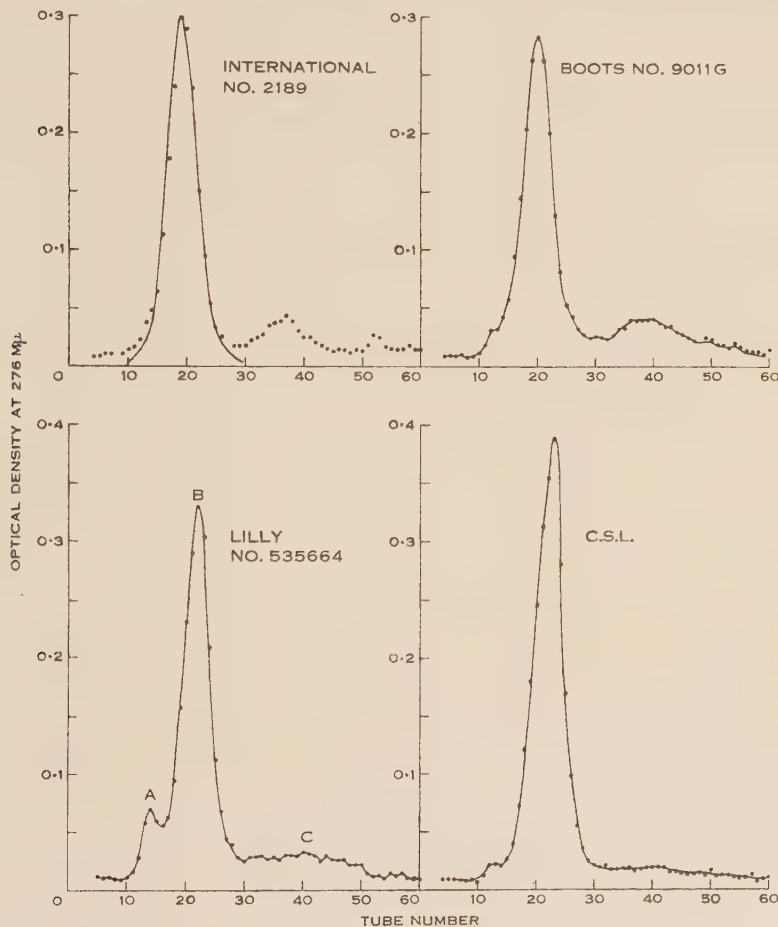


Fig. 1.—Chromatography of crystallized insulin samples on a 0.9 by 17 cm column of DEAE-cellulose at 25°C. About 10 mg of insulin was chromatographed in each case with 8M urea containing 0.01M Tris buffer and 0.001M "Versene" at pH 7.4. The effluent was collected in c. 0.7-ml fractions. Solid circles are experimental points. For International insulin No. 2189 only, the continuous line is the calculated theoretical curve.

### III. RESULTS AND DISCUSSION

Figure 1 shows the elution curves obtained by chromatographing four samples of insulin on DEAE-cellulose in buffers containing 8M urea at pH 7.4. The shoulders on the curves were reproducible. All the samples have separated into three



components, the percentages of each being given in Table 1. The values for the front A peak are only approximate due to difficulty in assessing the complete curve of the first component and hence are not identical with previous estimates (O'Donnell and Thompson 1960) where separation was more complete.

In the presence of 8M urea much lower concentrations of salt are required to elute the insulin from the DEAE-cellulose; for example, at 18°C and pH 7.4 in the absence of urea an ionic strength of approximately 0.31 was necessary to produce an  $R_F$  of 0.5 (O'Donnell and Thompson 1960) whereas in buffer containing 8M urea less than 0.014 ionic strength is required. The recovery of protein from the column was quantitative (90–100 per cent.) within the limits of error in fixing the base line.

TABLE I  
PERCENTAGE OF VARIOUS COMPONENTS IN COMMERCIAL INSULINS  
Insulin chromatographed on DEAE-cellulose at pH 7.4 in 8M urea containing  
0.01M Tris and 0.001M "Versene"

Component	Percentages of Components in Insulin			
	International No. 2189	Boots No. 9011G	C.S.L.	Lilly No. 535664
A	6	3	2	9
B	80	76	92	75
C	14	21	6	16

Our results are in general agreement with those of Harfenist and Craig (1952) who first showed that crystallized insulins could be resolved into native and desamido fractions by countercurrent distribution between *n*-butanol and aqueous dichloroacetic acid. Boardman (1959*b*) succeeded in partially resolving crystallized insulin by chromatography on a specially prepared sulphonated polystyrene stationary phase whereas Porter (1953) could not separate a desamido fraction by liquid-liquid chromatography on kieselguhr columns.

The separations by chromatography on DEAE-cellulose in buffers containing 8M urea are better than those obtained by either Porter (1953) or Boardman (1959*b*). Boots insulin No. 9011G has been examined by countercurrent distribution (Harfenist and Craig 1952) and chromatographically by Boardman (1959*b*), the latter reporting non-quantitative recoveries indicative of irreversible binding. The International standard insulin No. 2189 we have examined is supplied with sample curves obtained by the techniques of countercurrent distribution (Harfenist and Craig 1952) and liquid-liquid chromatography (Porter 1953) and the resolution we have obtained is superior to that shown by either of these techniques.

It is apparent that, of the insulins we have chromatographed, the C.S.L. insulin contains least of the minor components. However, the main peak shows a skewness not obvious in the other samples. Countercurrent distribution studies (Human and Leach 1960) by the method of Harfenist and Craig (1952) also showed less of the desamido component in a sample of C.S.L. insulin compared with either the Lilly or the International samples of insulin.

In the previous paper (O'Donnell and Thompson 1960) it was concluded that, in the absence of urea, the desamido fraction was inseparable from the main peak. From comparison with countercurrent distribution curves of samples of insulin (Harfenist and Craig 1952; Human and Leach 1960) it is probable that the C component reported here is the desamido fraction characterized by Harfenist and Craig (1952) and Harfenist (1953). The A component is the same as that previously separated by O'Donnell and Thompson (1960). A sample of desamido insulin isolated by countercurrent distribution from Lilly insulin (Human and Leach 1960) split, in the absence of urea, into component A and a component which ran in the same position as the major insulin fraction. With the buffer containing 8M urea at pH 7.4 a small sample of the desamido insulin split into only A and C components but insufficient material was available to give large unequivocal peaks.

The binding of proteins to ion-exchange resins is due to a combination of ionic, van der Waal's forces, and hydrogen bonds and the forces involved are usually multiple (Moore and Stein 1956). In the absence of urea relatively high concentrations of salt were required to elute the insulin but the presence of urea with its strong disaggregating properties and high dielectric constant reduced the binding of the insulin to such an extent that very little salt was required to elute the protein. Moreover, at pH 7.4 in the presence of urea, resolution of the desamido component (C) was achieved which was not possible in the absence of urea. Either the isoelectric points of the insulin components have been raised in 8M urea or the weaker binding of the proteins to the DEAE-cellulose is responsible for the improved resolution.

A theoretical distribution curve (Mayer and Tompkins 1947) agreed well with the experimental curve for the main peak (B) of insulin No. 2189 in Figure 1 which chromatographed with an  $R_F$  of 0.5. In order to compare the practical and theoretical insulin curves at a lower  $R_F$  value the effect of pH and temperature on the chromatography of insulin was investigated. A change of temperature between 10 and 35°C had no effect on the  $R_F$  in contrast to the marked effect in the absence of urea (O'Donnell and Thompson 1960). Increase in pH at 25°C to 8.3 lowered the  $R_F$  to 0.3 while at pH 8.9 it was 0.25. A theoretical curve for  $R_F = 0.3$  is given in Figure 2 (for insulin No. 2189) and it is seen that the chromatographic behaviour of insulin under these conditions is close to ideal. As the pH of the urea buffer was increased to pH 8.9 the separation of the minor components became scarcely detectable. This is because the desamido component (C) is not eluted at the higher pH value, while the minor A component becomes too diffuse for accurate measurement. Presumably the desamido component of insulin could be prepared by

adsorption on a DEAE-cellulose column at pH 9 under the conditions given here and subsequently eluted at a lower pH value.

At pH 6.4 the main peak is comprised of the A and B components and has shifted back towards the break-through point while the desamido component (C) appears as a pronounced shoulder on the elution curve (Fig. 3).

This trailing desamido component of the various insulins studied does not chromatograph the same in all cases (Fig. 1). For example, the Lilly insulin does

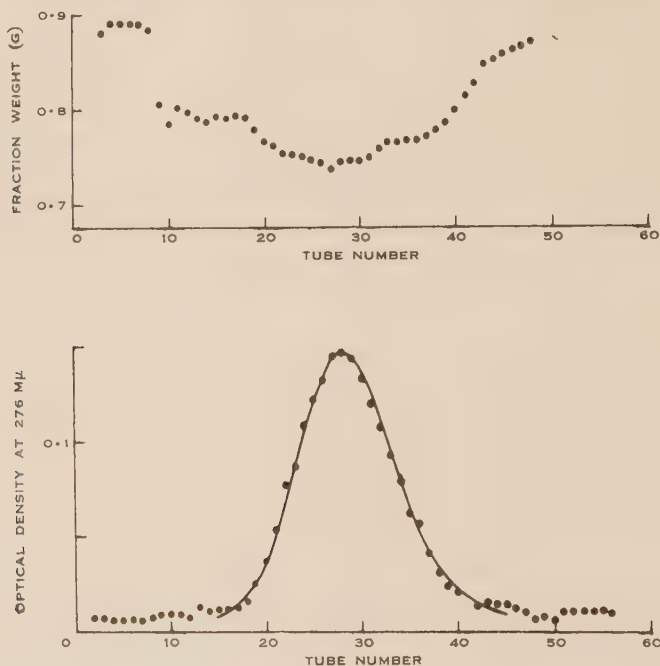


Fig. 2.—Chromatography of International insulin No. 2189 on a 0.9 by 14 cm column of DEAE-cellulose at 25°C. About 10 mg of insulin was chromatographed at pH 8.3 in a buffer containing 8M urea, 0.01M Tris, and 0.001M "Versene". The effluent was collected in c. 0.7-ml fractions. Solid circles are experimental points and the continuous line is the calculated theoretical curve. Weights of fractions (12 drops) are given in the upper curve.

not show a pronounced peak as do the Boots and International insulins and maybe more than one type of deamidated molecule is present. It is possible that the deamidation of insulin has occurred at different positions on the molecule. There are six amide groups in insulin and some deamidation of the terminal asparagine residue has been reported (Sanger and Thompson 1953; Harris 1955). In addition to peptides containing the terminal asparagine in a deamidated form some peptides have been isolated, indicative of deamidation of a glutamine residue (Sanger, Thompson, and Kitai 1955). If a mixture of various deamidated components is present this might not be expected to chromatograph as a single substance.

The presence of a desamido fraction in commercial insulins suggests that either deamidation has resulted from manufacturing processes or that synthesis of a proportion of deamidated molecules has occurred in the pancreas (Vaughan and Steinberg 1959). The variation in percentage of the desamido fraction between the different insulins might suggest that the former explanation is more likely. However, it is

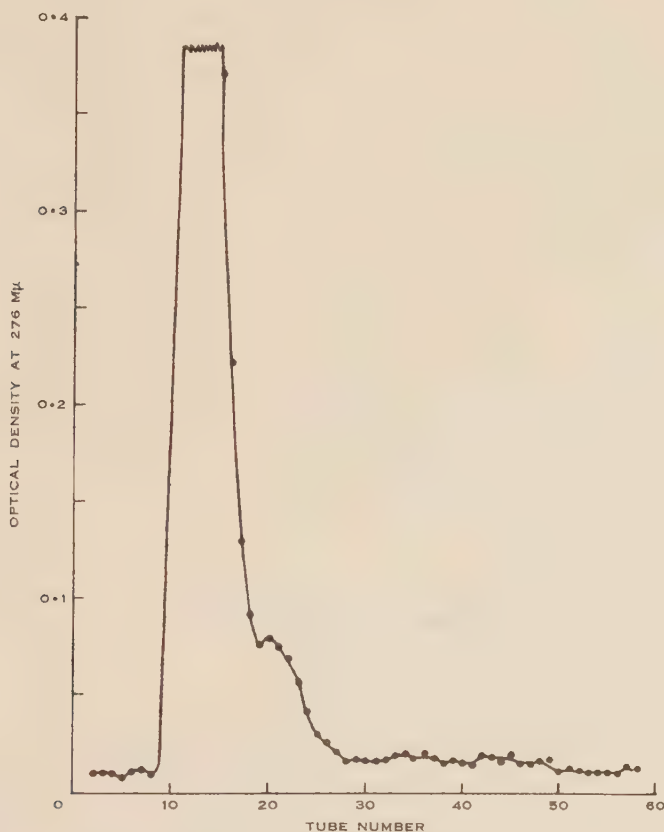


Fig. 3.—Chromatography of International insulin No. 2189 on a 0.9 by 17 cm column of DEAE-cellulose at 25°C. About 10 mg of insulin was chromatographed at pH 6.4 in a buffer containing 8M urea, 0.01M Tris, and 0.001M "Versene". The effluent was collected in c. 0.7-ml fractions.

also possible that the various manufacturing processes fractionate the desamido component from the bulk of the insulin to different extents. Moreover, studies on ribonuclease (Martin and Porter 1951; Hirs, Moore, and Stein 1953; Aquist and Anfinsen 1959) have shown the presence of at least two components, differing probably by only one carboxyl group (Tanford and Hauenstein 1956), in crude extracts of pancreas under conditions which did not seem to favour deamidation of one molecule into the other.



As Moore and Stein (1956) have pointed out, the number of proteins that have been chromatographed successfully by elution analysis at constant pH and ionic strength is limited to a comparative few, e.g. cytochrome *c*, ribonuclease, lysozyme, chymotrypsin, and chymotrypsinogen and most of these have been run on columns of "Amberlite IRC-50", where a reversible distribution coefficient giving an  $R_F$  between 0.7 and 0.1 has been obtained. There are some proteins such as the haemoglobins studied by Boardman and Partridge (1955) which can be chromatographed at constant pH and ionic strength but only at relatively high  $R_F$  values ( $>0.5$ ). At lower  $R_F$  values the adsorption is not truly reversible as it is in the case of other proteins such as cytochrome *c* (Boardman 1959*a*) which can be chromatographed at low  $R_F$  values where the test for homogeneity of a protein by comparison of experimental and theoretical curves is more exacting. Insulin in buffers containing 8M urea appears to undergo a true reversible adsorption on DEAE-cellulose at an  $R_F$  of 0.3 as shown by the close agreement between the theoretical and practical curves (Fig. 2).

#### IV. ACKNOWLEDGMENTS

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# THE PHYSIOLOGY OF GROWTH IN THE WHEAT PLANT

## I. SEEDLING GROWTH AND THE PATTERN OF GROWTH AT THE SHOOT APEX

By R. F. WILLIAMS\*

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### *Summary*

Seedling growth of wheat in a constant environment is studied over a period of 21 days. Dry weights of leaves, leaf sheaths, stem, and roots are given for 11 occasions. The pattern of dry weight change is also presented in terms of the changing ratios of plant parts.

Growth rates of leaf primordia are determined in terms of volume change based on the technique of serial reconstruction.

For an 11-day shoot apex, a detailed account is given of cell-size distribution along the leaf primordia and within the apex itself. It is estimated that, prior to the onset of cell enlargement, the mean cell-generation times for the young leaf primordia range from 12 hr to 3 days.

An integrated picture of the early growth of the primary shoot is attempted, mainly in terms of the concept of relative growth rate. The rates for leaves and roots are particularly high while seed reserves are available. There is a progressive change in dominance from leaf growth to stem growth. Early growth of each leaf primordium is exponential, but the exponent decreases with leaf number in a rather discontinuous manner. Following the exponential phase, the rates rise to maxima and then fall asymptotically to zero.

It is suggested that intra-plant competition for energy substrates may play an important role in determining the pattern of development of the primary shoot of wheat.

### I. INTRODUCTION

Precise quantitative information on both structural and functional attributes of plant growth is required if we are to have an understanding of the internal factors and mechanisms which integrate the parts of a plant into the whole organism. This was clearly recognized in ontogenetic studies of wheat and Sudan grass by Ballard and Petrie (1936) and Petrie (1937), of oats by Williams (1936, 1938, 1948), of tobacco by Petrie, Watson, and Ward (1939), Watson and Petrie (1940), and Petrie and Arthur (1943), of flax by Tiver (1942), and of linseed by Tiver and Williams (1943). More recently, studies on similar lines have been made for barley and rye by Williams and Shapter (1955) and for the tomato plant by Gates (1955*a*, 1955*b*, 1957). Common features of all these studies are adequate sampling on from five to ten occasions during growth, the separation of the plants at least into their major parts, and the analysis of growth in terms of dry weight change. To greater or lesser extents, too, these studies have included chemical work sufficient to build up a picture of the intake and distribution of certain essential elements, particularly nitrogen and phosphorus.

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The extraction of general principles relevant to the physiology of growth from complex sets of data such as the above is not easy, and has been attended by only partial success when attempted by Watson and Petrie (1940) and by Williams (1955). Perhaps the most significant positive contribution has been their emphasis upon competitive demand within the plants for metabolites and nutrients alike. Differences in growth pattern induced by differential nutrition and other treatments were seen as a consequence of such competition, for the growth of a given organ was found to be stimulated, relative to other organs, if it was nearer the source of a deficient nutrient or metabolite. Bald (1946) considered the principle of intra-plant competition to be adequate for the interpretation of differences in growth form, maturity, and yield between varieties and strains of the potato plant. It is also significant that zoologists have been thinking along similar lines, for Spiegelman (1945) developed this theme on quite a broad basis, and gave it mathematical form. His examples were drawn mainly from coelenterate hydroids, which possess some characteristics in common with plants. Thus they are normally anchored to their substratum, and they possess apical dominance which is rather similar to that in plants.

It is now accepted that the early development of successive organs is quite as significant for our understanding of plant growth as is the later and more obvious unfolding of these organs. Nevertheless, our knowledge of the quantitative changes during early growth is very limited, and much might be gained by measuring rates of growth of vegetative organs from the time of their initiation through to maturity and senescence. This is the main purpose of the present study, and the wheat seedling was chosen as the test object because a great deal is already known about this plant, and because further information about it is likely to be relevant to other cereals and to many gramineous pasture plants. It was necessary to restrict the work initially to the quantitative description of growth in one controlled environment. Dry weight changes in the first four leaves, the stems, and the roots were determined from the time that these parts could be separated with reasonable precision. For all smaller structures, the technique of serial reconstruction was adopted, and growth determined as volume change with time.

It was Wilhelm His who drew attention to the importance of measurement for the understanding of morphogenetic processes, and it is to him that we owe the procedure of serial reconstruction for the understanding of embryonic structure and development. In one place His (1888) says:

"The ways of determining the forms and volumes of germs and embryos are somewhat longer and more tiresome than the simple inspection of stained sections; but the general scientific methods of measuring, of weighing, or of determining volumes cannot be neglected in embryological work, if it is to have a solid foundation of facts, for morphologists have not the privilege of walking in easier or more direct paths than workers in other branches of natural science."

While serial reconstruction has been used over and over again for the description of form changes in embryos and embryonic organs in animals and plants, there seem to be no recent examples of its use in any precise quantitative sense. The present paper attempts this task for the shoot apex of the wheat plant.



## II. EXPERIMENTAL PROCEDURE

(a) *Plant Culture, Sampling, and Dissection*

A spring wheat (*Triticum aestivum* L., cv. Nabawa) was grown in a constant environment of which the temperature was 20°C and the light intensity was approximately 950 f.c. at plant level. The grain was set to germinate in petri dishes and, after 24 hr, was sown in 10-oz cans filled with vermiculite. During the course of the experiment the pots were flushed through daily or more often with Hoagland No. 2 nutrient solution.

As they matured, leaves 1 and 2 tended to develop chlorotic areas near their tips. Later experience suggests that the use of half-strength instead of full-strength nutrient would have eliminated this condition. The plants also suffered a mild water stress for a short time on day 20.

The replication was five throughout, but the number of plants per can varied with the age to which they were grown. In the first experiment there were 12 grains or seedlings per replicate for days 0, 1, and 6; 10 plants per replicate for days 8 and 11; 8 per replicate for days 13 and 15; and 6 for days 18 and 21. In the second experiment there were 12 grains or seedlings per replicate for days 0, 1, 2, 3, 4, and 8.

As plant dissections had to be extended over a considerable period on each sampling occasion, the replicates were always drawn in the same order from blocks of cans which had been sown at 75-min intervals. In this way it was possible to ensure that the age at time of dissection of each seedling was within 35 min of the day specified.

At day 1, dissection was limited to the separation of the embryo (without scutellum) and the rest of the grain. The coleoptile and the roots were first separated on day 2, leaves 1, 2, 3, and 4 were first separated on days 3, 6, 8, and 15 respectively, and leaf sheaths 1, 2, and 3 on days 8, 11, and 18 respectively. The roots were cut at their points of emergence from the coleorhiza, but their dry weights were later adjusted to include an estimate of the weight of the stumps within the coleorhiza. Leaves were separated at their bases or at the ligule as soon as this was present. The stem fraction was the least satisfactory in that all organs too small to be dissected off were included with it. Some adjustments were made (see Table 1), and it should be noted that tillers contribute appreciably to stem dry weight after day 15.

All parts were dried at 80°C in an oven with forced draught.

(b) *Volume Integration*

Plants additional to those used for dissection and dry weight determinations were sampled for the serial reconstruction studies. The whole embryo at day 1, or that part of the primary shoot which contained the apex and the younger leaf primordia, was fixed in formalin-acetic-alcohol. Acid fuchsin was added to the fixative to facilitate dissection prior to embedding in wax. For each of 11 sampling occasions, transverse serial sections of four axes, and longitudinal sections of one axis were cut at 10  $\mu$  and stained in iron alum haematoxylin and erythrosin.



Volume estimation of irregular solids can be made from equally spaced sectional areas, preferably taken along the major axis of the solid, and this is the principle underlying serial reconstruction. The areas of about 15 sections were determined for the larger primordia, but all sections were usually determined for small structures. Areas were determined from photographic enlargements, like those illustrated in Plate 2, by superimposing a centimetre grid (on glass), and counting squares and tenths of squares. Even with such irregular shapes, it was found that duplicate determinations with different grid positions agreed very closely. Volumes were determined by a graphical procedure which is illustrated in Figure 1 for an 11-day

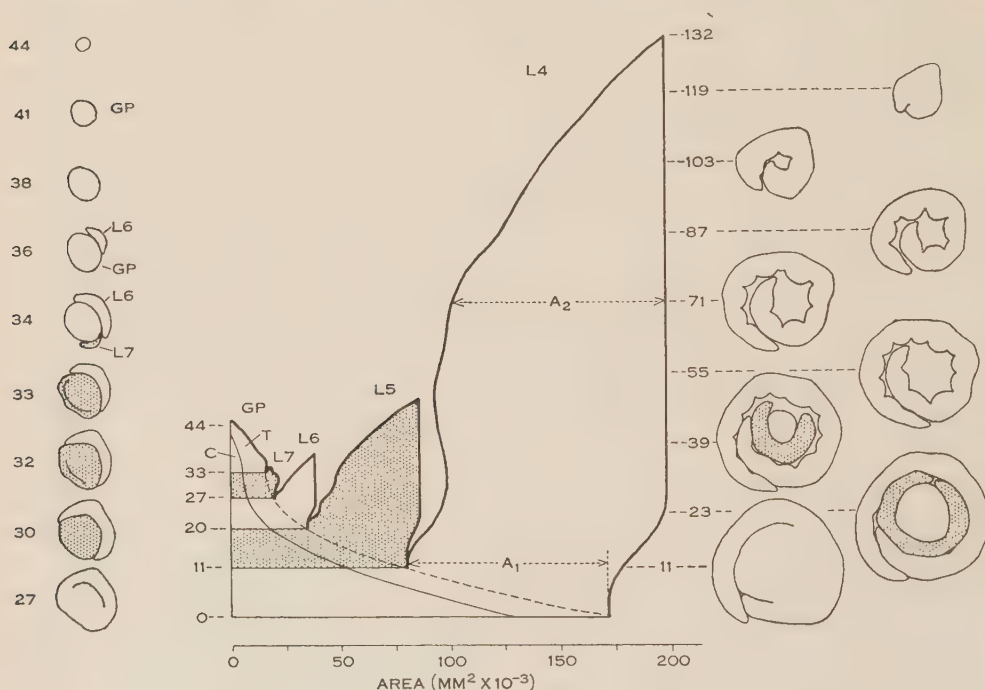


Fig. 1.—Diagram illustrating the graphical procedure used for determining the volumes of leaf primordia and internodal tissues of the shoot apex. For full explanation see text. GP, growing point; T, tunica; C, corpus; L4, L5, etc., successive leaf primordia. Diagram based on an 11-day apex, 1.32 mm long.

seedling axis. Outline drawings of some of the sections used for leaves 4 and 5 are shown to the right of the diagram, and others for the apex and leaves 6 and 7 are to the left, together with their section numbers. It was necessary to set vertical limits to the stem tissue associated with successive primordia, so the upper limit was defined by that section in which the primordium appeared half united with the axis (e.g. sections 11, 27, and 33 of Fig. 1). The stem areas at these points were taken as those of the first complete stem sections immediately above. Another problem was to provide an objective definition of the inner limit of the leaf primordium. The work of Barnard (1955) is relevant here, for he has shown that leaf primordia in wheat arise by the periclinal division of cells of the tunica, the corpus contributing nothing

to their development. The histological pattern of the vegetative apex in an 8-day seedling is shown in Plate 3. In longitudinal section, the two-layered tunica is clearly distinguishable from the corpus, and the thickness of the tunica was found to be remarkably constant throughout the period of the experiment. This fact was used in defining areas appropriate to the tunica, *T*, and the corpus, *C*, in Figure 1, and the same convention was extended to the partitioning of the stem into portions deriving from the tunica and corpus respectively.

The partial areas of the central diagram of Figure 1 are directly proportional to the volumes of the structures they represent, and the total volume has in effect been divided into major units made up of a primordial leaf lamina (the ligule and leaf sheath arise at a later stage), and two stem portions. Together these are equivalent to the *growth units* of Sunderland and Brown (1956). However, for studies of the growth rates of leaf primordia, it is desirable to include all tissues derived from the tunica and not to restrict measurements to that part of the primordium which projects from the stem. At least in the present case, such restriction would have introduced large positive errors into estimations of relative growth rate in very young primordia. In what follows, a leaf primordium is defined as the lamina plus the outer or tunica-derived part of the associated stem tissue. The growth rates of the corpus-derived tissues, or pith, also become more meaningful as a result of this procedure.

The present method of serial reconstruction would be suspect if it could be shown that there had been appreciable differential shrinkage or distortion of the parts measured. It seems inevitable that there should be some shrinkage during fixation, but obvious distortion of cell walls was not found (see Plate 2), except in more mature tissues than those actually measured. Compression and distortion during cutting and mounting were thought to be more likely sources of error and were examined accordingly. For several ages of material, measurements made first on the face of the wax block and then on the mounted section indicated an area reduction of  $18.7 \pm 1.6$  per cent. Although this reduction is rather large, its relative constancy indicates that relative rates of volume change would be little affected by it.

Only one axis for each of the 11 occasions was examined in the detailed manner of Figure 1; for the other three axes, the volumes of all but the smallest primordial laminae were estimated from the regressions of Figure 2. Stem and apical volumes were determined directly for each axis. In the regressions, actual volume (direct method) is expressed as a function of the product of total length (132 sections or 1.32 mm for L4 in Fig. 1) and the sum of  $A_1$  and  $A_2$ , where  $A_1$  is the basal area (by difference of the stem areas) and  $A_2$  is the cross-sectional area half way up the free part of the lamina. Equation (1) refers to L1 and L2 alone, and equation (2) refers to L3–L9 inclusive. Although the separate equations were used in what follows, there is a good case for pooling all the values to give the following equation:

$$Y = -0.3066 + 0.8646X + 0.0407X^2,$$

where  $Y$  is the logarithm of the actual volume  $V$ , and  $X$  is the logarithm of  $V'$  as defined in Figure 2.

## (c) Cell Size

The determination of cell-size distribution along the lengths of the primordia and the pith was attempted for one 11-day axis only. Appropriate sections were projected, and all nuclei or recognizable fragments of nuclei were counted. Since the mean length of the nuclei, as seen in longitudinal section, was  $9\ \mu$  and the thickness of the sections  $10\ \mu$  it follows that there would be 10 whole nuclei per section for every 19 counts by this method. Knowing the volume of the whole section, it was possible to compute the mean cell size for that section.

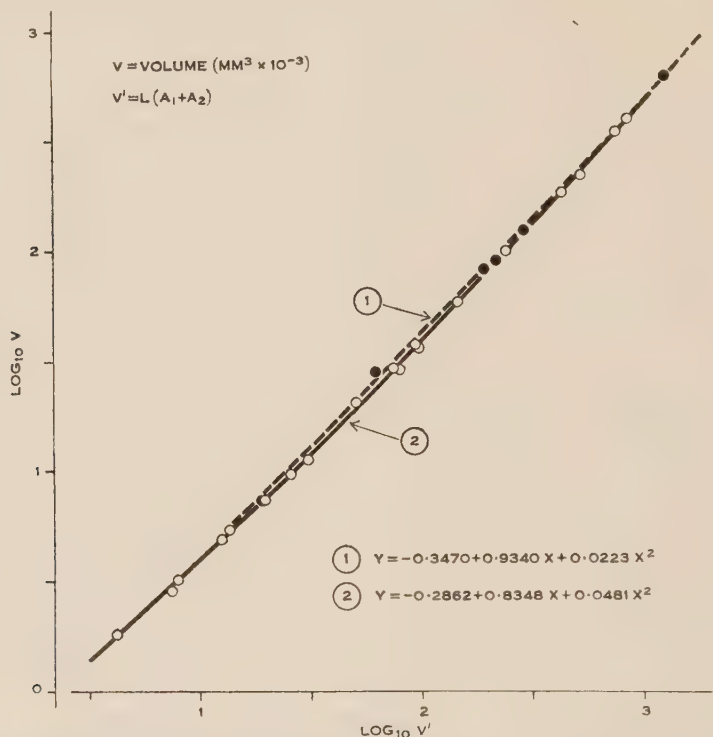


Fig. 2.—Regressions used for determining the volume  $V$  of a leaf primordium from its length and two sectional areas. Equation (1) for  $L1$  and  $L2$ ; equation (2) for  $L3$ – $L9$  inclusive. See text for further explanation.

## III. PRESENTATION OF DATA

## (a) Dry Weight Change

The dry weights for the main categories of plant parts, are presented in Table 1 and Figure 3. For the latter the values are plotted additively, so that the upper curve describes the growth of the whole organism, including the grain. The coleoptile has been regarded as a leaf sheath, and the coleorhiza included with the stem. The dry weights of individual leaves and leaf sheaths, and of the coleoptile are presented in Tables 2 and 3 and in Figure 4. This figure also shows the dry weight changes of the grain and the roots to the same scale.

The justification for uniting the data for the two experiments will be found in the parallel sets of values for day 4 (see Tables 1, 2, and 3); similar sets for day 8 showed equally good agreement. There is, however, an initial difference of 4 mg in mean grain weight for the two experiments, and this is reflected in the pairs of values for rest of grain (Fig. 4) for days 1, 4, and 8. The lower values in each case refer to the first and longer experiment, the upper values refer to the second experiment, which was done to fill in the detail for the first 4 days of growth.

From Figure 3, two distinct phases of growth are apparent. In the first, dry weight increase in the young seedling is dependent on grain reserves, and in the second the seedling is self supporting. There is, however, some overlap of these

TABLE 1  
DRY WEIGHTS (MG PER PLANT) OF WHOLE PLANT AND PRINCIPAL PARTS

Expt.	Day	Leaves	Leaf Sheaths	Stem	Roots	Total without Grain	Rest of Grain	Whole Plant
2	0	—	—	—	—	—	—	55.78
2	1	0.06*	0.15*	0.38*	0.18*	0.77	54.29	55.06
2	2	0.21†	0.38	0.51†	0.72	1.82	52.21	54.03
2	3	0.75†	1.16	0.66†	2.58	5.15	48.36	53.51
2	4	2.15†	2.16†	0.69†	5.24	10.24	42.12	52.36
1	4	(2.36†)	(2.17†)	(0.64†)	(5.51)	(10.68)	(39.66)	(50.34)
1	6	8.76†	3.58†	0.73†	10.09	23.16	26.09	49.25
1	8	21.57	5.30†	1.07†	12.84	40.78	13.15	53.93
1	11	40.34†	8.99	1.77†	13.90	65.00	5.29	70.29
1	13	53.01†	11.95†	3.12†	15.61	83.69	6.12	89.81
1	15	73.70	17.68†	5.68†	19.56	116.62	4.93	121.55
1	18	106.01	22.28	27.73	30.68	186.70	4.74	191.44
1	21	123.84	30.41	53.29	42.55	250.09	4.83	254.92

\* Estimated from total embryo weight and volumes of parts (see text and Fig. 4). In addition, a constant correction of 0.21 mg. was made to the roots and deducted from the stem fraction from day 2 onwards, this being the estimated weight of root stumps within the coleorhiza.

† Corrected to include (for leaves and leaf sheaths) or exclude (for stems) the estimated dry weights of parts too small to be dissected, but of known volume. Values in parenthesis in Tables 1, 2, and 3 establish the link between the two experiments.

phases. Thus the weight of the whole organism begins to increase after day 6, suggesting that gain from photosynthesis had begun to exceed respiratory losses. On the other hand, weight losses from the grain continue until some time between day 8 and day 11. What is not obvious from Figure 3 is that the relative growth rate,  $R$ , of the seedling is much higher in the first than in the second phase of growth. By plotting the same data on a logarithmic scale, this is shown quite clearly (see Fig. 5, second curve from top). The mean values of  $R$  for days 1–4 and 8–18 are 0.862 and 0.151 g/g/day respectively. The transition from the higher to the lower rate takes about 4 days (day 4–day 8). The still lower rate for days 18–21 (0.099 g/g/day) can perhaps be attributed to the period of water stress suffered on day 20 (see Section II(a)).



An  $R$  value of 0.151 for a self-supporting grass seedling suggests that growing the test plants in continuous light of rather low intensity was not detrimental to growth. Mitchell (1956) obtained maximal  $R$  values of about 0.16\* for a number of grasses grown at about the same temperature but with only 12 hr of light of a much higher intensity (2700 f.c.). The highest values of  $R$  obtained by Ballard and Petrie (1936) for wheat, and by Williams (1936) for oats grown in a glass-house, were 0.08

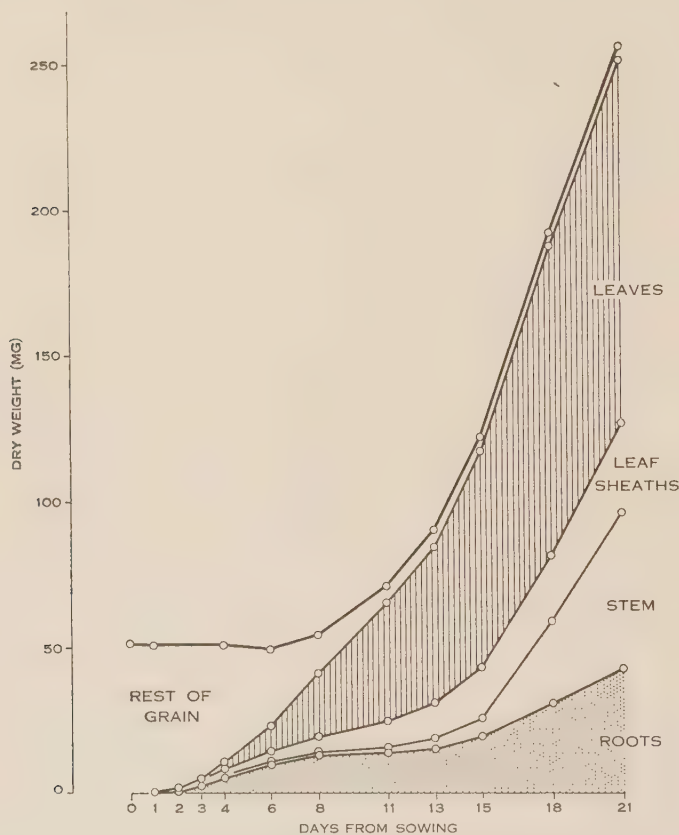


Fig. 3.—Dry weights of principal plant parts plotted additively for early seedling growth in wheat.

and 0.14 respectively. Their day temperatures were reasonably comparable, but night temperatures were low.

Root growth was highly correlated with the two phases of growth. The growth of the five (or six) primary roots was dependent mainly on grain reserves, and dry weight increase of the root system almost ceased between days 8 and 11 (Fig. 4). Growth in the second phase was by adventitious roots and by fine branching of the primary roots.

\* These were for shoots only. The shoot value for the present experiment was 0.17.

The growth of the coleoptile and of the first leaf blade seem to have depended mainly on grain reserves. However, the first leaf emerged from the coleoptile  $3\frac{1}{2}$  days after sowing, and would soon contribute to dry matter production.

TABLE 2  
DRY WEIGHTS (MG PER PLANT) OF INDIVIDUAL LEAVES

Expt.	Day	Leaf 1	Leaf 2	Leaf 3	Leaf 4
2	3	0.72	—	—	—
2	4	2.08	—	—	—
1	4	(2.30)	—	—	—
1	6	8.29	0.45	—	—
1	8	17.62	3.84	0.11	—
1	11	20.92	17.80	1.59	—
1	13	20.05	24.82	8.04	—
1	15	20.83	29.16	23.07	0.64
1	18	21.68	30.17	46.49	7.67
1	21	20.90	29.98	49.52	23.44

As has already been shown, the comparison of growth rates in time is greatly helped by examining the data on a logarithmic scale. This also holds for the comparison of the rates of growth of different organs at the same time (see Fig. 5).

TABLE 3  
DRY WEIGHTS (MG PER PLANT) OF THE COLEOPTILE AND OF INDIVIDUAL LEAF SHEATHS

Expt.	Day	Coleoptile	Leaf Sheath 1	Leaf Sheath 2	Leaf Sheath 3
2	2	0.38	—	—	—
2	3	1.16	—	—	—
2	4	2.13	—	—	—
1	4	(2.13)	—	—	—
1	6	3.35	—	—	—
1	8	3.69	1.58	—	—
1	11	3.49	4.99	0.51	—
1	13	3.53	4.90	3.49	—
1	15	3.44	5.67	8.07	—
1	18	2.82	6.25	9.89	3.31
1	21	2.73	5.98	10.78	10.92

Unfortunately, the only separation that could be made at day 1 was for embryo and rest of grain. However, it is possible to estimate the dry weights from the volumes of the main parts. The assumption was made that, at this early stage, dry weights are proportional to the volumes occupied by the various parts. Figure 6 was built up by serial reconstruction as in Figure 1, but for the whole embryonic axis, so that areas within the diagram were proportional to volume. The volumes of the parts are listed beside the diagram, and their dry weights were estimated from these

and the weight of the whole embryo (0.77 mg). The resulting values are included in Table 1 and their logarithms in Figure 5. Further corrections, also based on the volume studies, are incorporated in Table 1 and Figure 5. Tables 2 and 3 report only the weights of parts actually dissected.

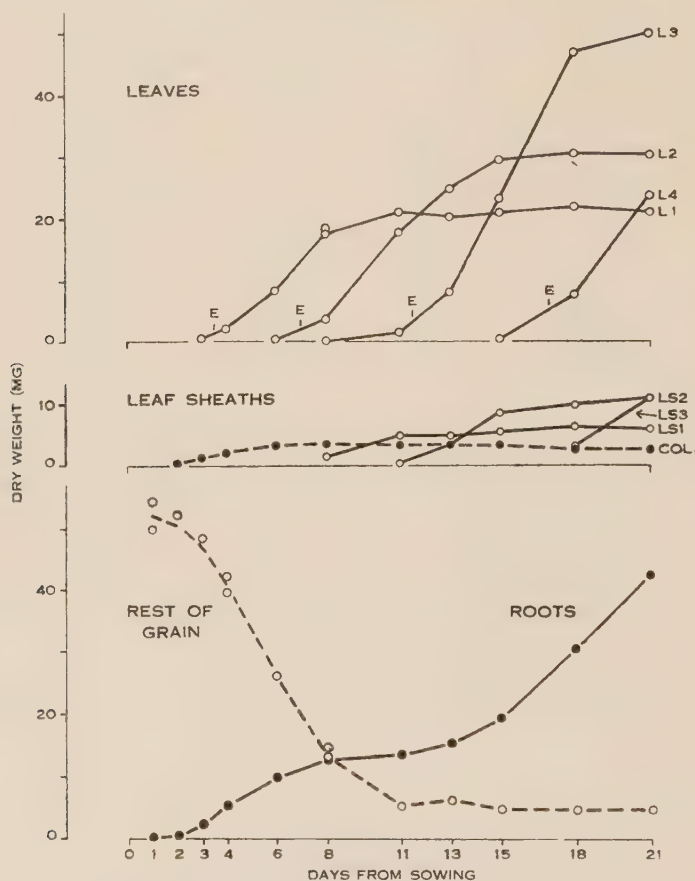


Fig. 4.—Dry weights of individual leaves and leaf sheaths, and of roots and grain for early seedling growth in wheat. *E*, times of emergence of successive leaves.

Differences in the slopes of the curves for coleoptile, leaves, stem, and roots (Fig. 5) imply quite marked differences in their relative growth rates during the first phase of growth. Numerical values of  $R$  for days 1–3 were as follows:

coleoptile	1.03	stem	0.28
leaves	1.22	roots	1.32

Root growth was fastest at first but soon fell to a very low rate ( $R = 0.08$  for days 8–11). Coleoptile growth also fell very quickly and ceased at day 6. By contrast, the relative growth rate of the leaves fell more slowly and settled down to a steady rate ( $R = 0.138$  for days 11–18). In so doing, the leaves became much

the largest part of the plant. The true leaf sheaths could not be effectively separated before day 8 and, as might be expected, their rate of growth closely parallels that of the leaves. The part called stem here might perhaps have been called "rest of plant", for its relative growth rate for days 1-3 was probably dominated by that of the

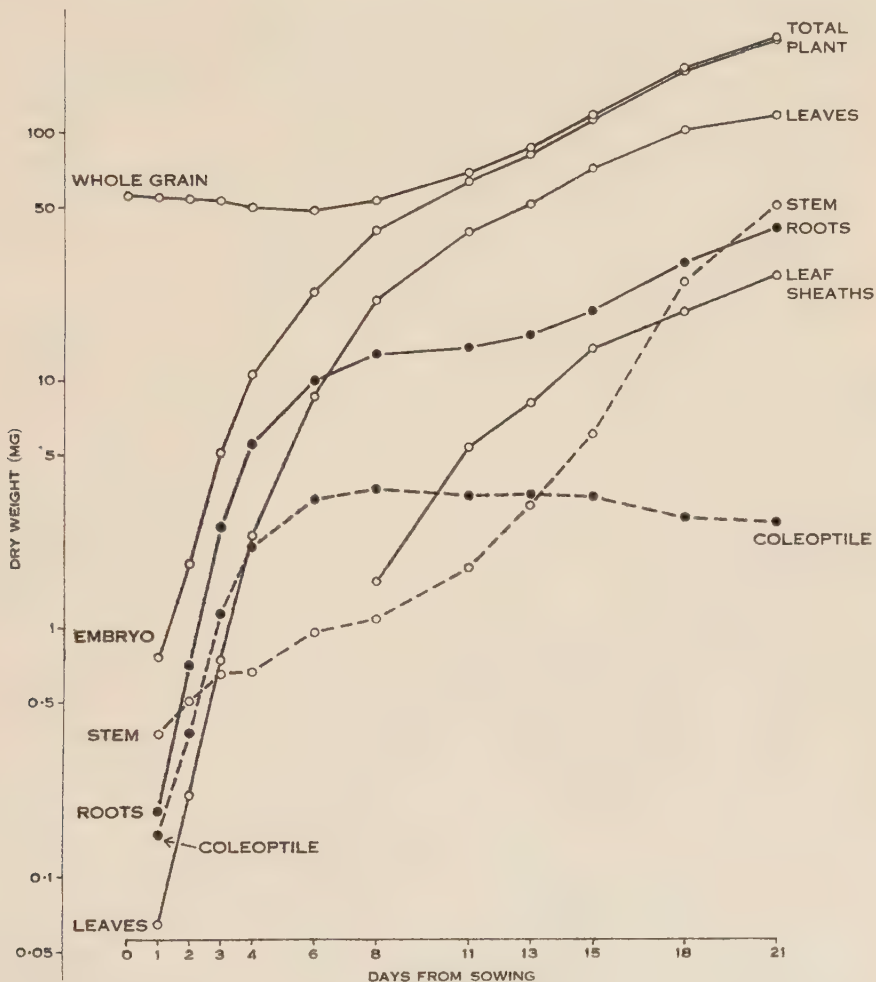


Fig. 5.—Dry weights (logarithmic scale) of principal plant parts and of total plant for early seedling growth in wheat.

coleorrhiza, and after day 15 it was increased by the inclusion of tillers. The rather high rate for the mid period ( $R = 0.313$  for days 11-15) can reasonably be attributed to the stem itself.

#### (b) Derived Data

Many papers on plant growth and nutrition have presented ratios of the dry weights of the shoots to roots as a means of defining the effects of treatment on



structural change. A better picture is obtained when such ratios are referred to the total plant weight, for the plant can be divided into any required number of parts, and the ratios plotted additively to give a unified picture. This has been done for the present experiments in Figure 7. It should be noted, however, that the parts have here been separated on a strictly morphological basis, so that the leaf weight ratio is based on all leaf laminae, irrespective of whether they were exposed to the light or not.

The leaf weight ratio increased from the small value of 0.08 (day 1) to a maximum of 0.63 (day 13). The subsequent fall in this ratio would have been less

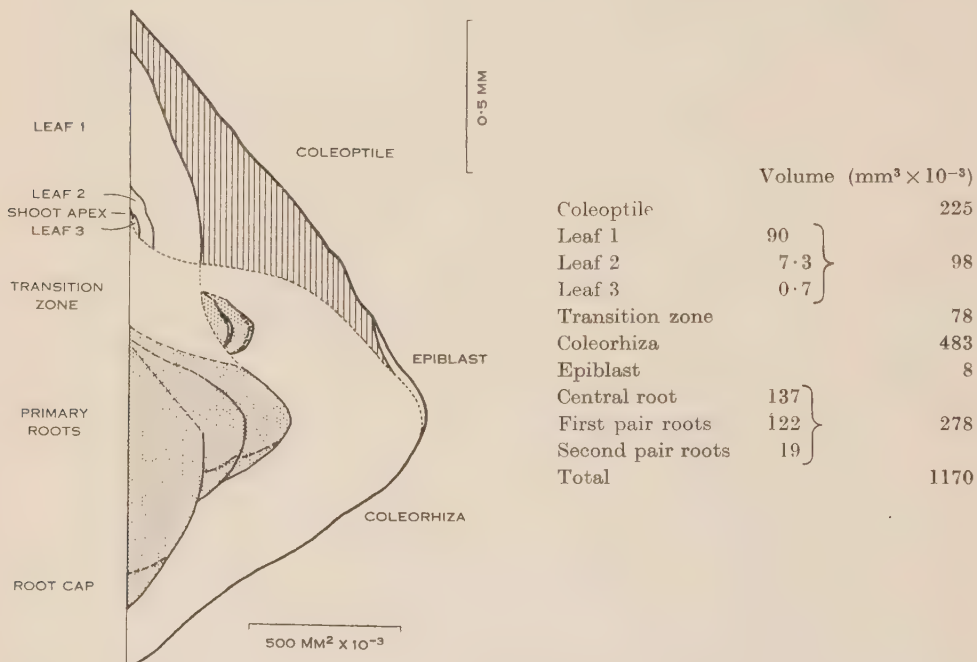


Fig. 6.—Volume distribution in embryo (without scutellum) after soaking the grain for 24 hr.

pronounced if tiller leaves had been included for days 18 and 21. The leaf-sheath ratio (including coleoptile) was rather constant throughout the experiment. The root weight ratio rose rapidly from 0.24 (day 1) to 0.50 (day 3) and then fell more slowly to 0.17 (day 15). The high initial value of 0.49 for the stem weight ratio is a reflection of the fact that the stem fraction was then made up of the transition zone, the coleorhiza, and the epiblast (Fig. 6). These grew rather slowly at first, so the stem weight ratio fell to only 0.03 by day 6. The sudden increase after day 15 was due to the growth of tillers.

The distribution indices of Figure 8 provide a descriptive explanation of the changes with time in the weight ratios of Figure 7. Distribution indices are obtained by expressing the increments in dry weight of leaves, roots, etc. for each interval as percentages of the total dry weight increment for that interval. Where, as in this

case, such indices are available for a succession of harvest intervals, they give quantitative expression to the changing growth pattern (Williams and Shapter 1955).

Figure 8 shows that, for the first few days, more than 50 per cent. of the dry matter from the grain was used for root growth, and only about 15 per cent. for leaf

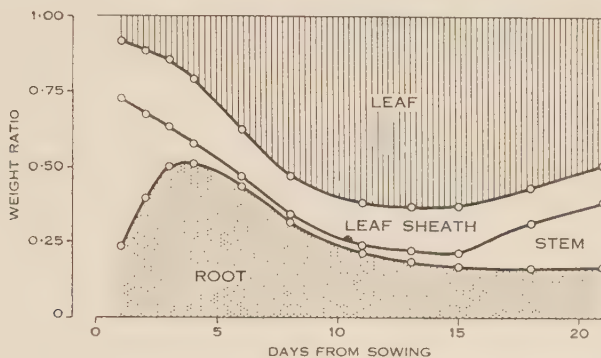


Fig. 7.—Dry weight ratios of principal plant parts plotted additively for wheat seedlings.

growth. However, by the time that grain reserves were exhausted (days 8–11) the indices were more than reversed, for the roots were then getting only 4 per cent., and the leaves 78 per cent. of the dry matter increase. Thereafter the root index increased and the leaf index decreased fairly slowly.

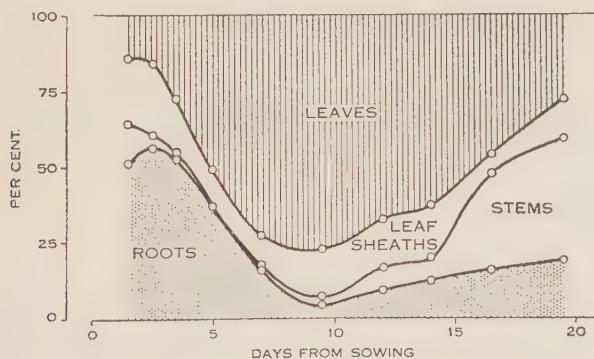


Fig. 8.—Indices of distribution of dry matter between the principal plant parts of wheat seedlings.

### (c) Volume Change

The primary data for volume changes at the shoot apex are presented descriptively in Figures 9 and 10. For each of the 11 occasions, a volume-distribution diagram is shown complete, but on a small scale, at the right; at the left is shown the detail in the region of the apex. The diagrams were compounded from mean lengths and areas for the four replicates at each occasion. In interpreting them, it

should be borne in mind that they present spatial arrangement in a rather schematic way. At the same time they do provide a useful bridge to the still more abstract treatment of Figure 11.

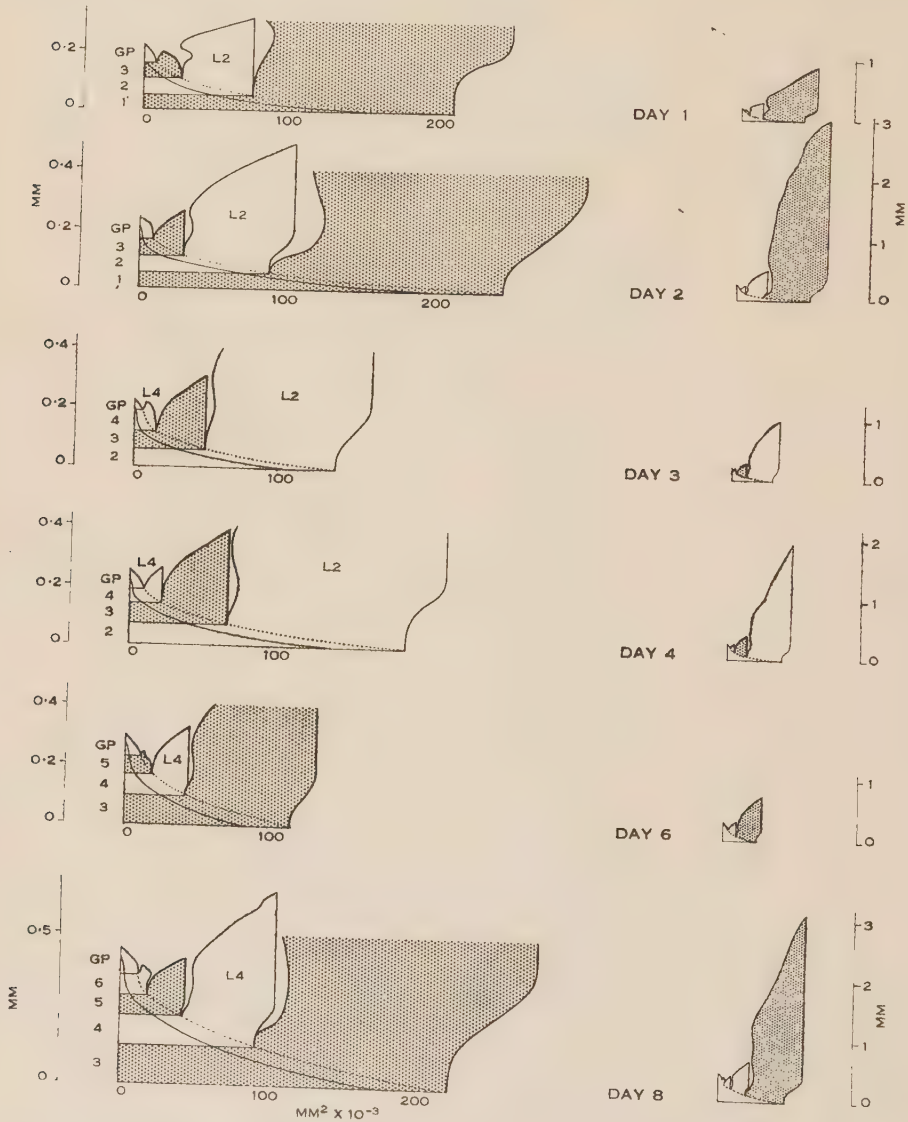


Fig. 9.—Volume distributions within shoot apices of the ages shown. GP, growing point; 1, 2, 3, successive internodes; L2, L4, leaf primordia.

Three leaf primordia are preformed in the grain (see day 1) and new primordia appeared at intervals of rather more than 2 days under the conditions of the experiment. The ninth, and last leaf primordium was already present on day 15, and the apex began to elongate prior to spike formation. The change from vegetative to

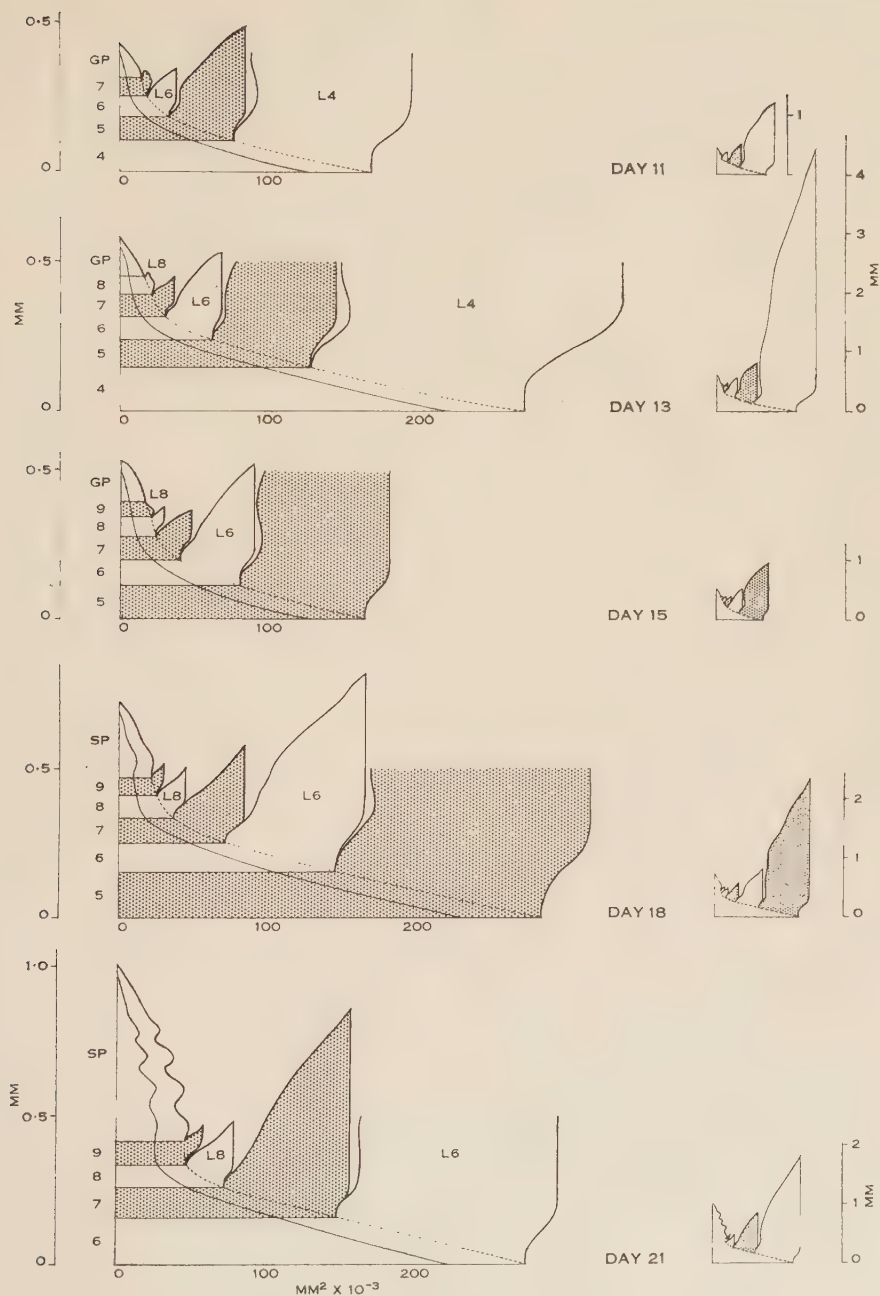


Fig. 10.—Volume distributions within shoot apices of the ages shown. *GP*, growing point; 4,5,6, successive internodes; *L4*, *L6*, leaf primordia; *SP*, spike primordium.



reproductive development gains expression in a number of ways, all of which imply a shift in dominance from foliar to cauline structures. The growth rates of the earlier leaf primordia are very high indeed (Fig. 9), the highest being a fourfold increase in leaf one (L1) from day 1 to day 2. Even L3 doubled its size over the same period. For the interval day 18–day 21, however, the fastest rate is for L6

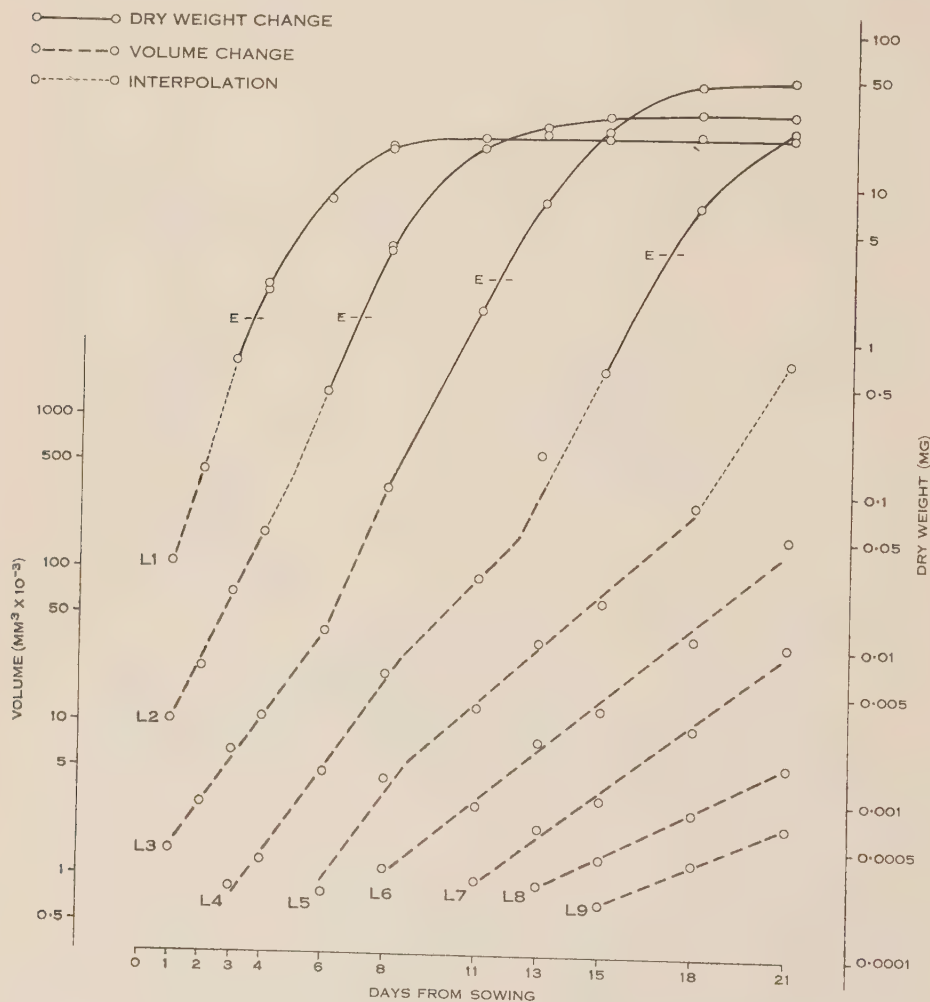


Fig. 11.—Dry weights of leaves and volumes of leaf primordia plotted on a common logarithmic scale. *E*, times of emergence of the successive leaves.

(Fig. 10), and this reduces to a 75 per cent. increase per day. Leaves L8 and L9 increased little more than 20 per cent. per day over the same period.

That there is a progressive increase in apical growth as such is clear from Figures 9 and 10, for the apical dome grows further and further away from the site of initiation of the most recent leaf primordium. However, it is not easy to get a completely satisfying measure of this change. The line drawings at the foot of Plate 3

also demonstrate the change, and show that the cauline part of the apex changes from a rather flat cone to an acute one, and that the apical dome grows further and further away from the apex of this cone. This suggests that the height of the dome above a given stem sectional area (e.g.  $100 \text{ mm}^2 \times 10^{-3}$ ) might be a suitable index of this aspect of apical growth.

Figures 9 and 10 show also that there is a progressive change with leaf position in volume distribution along the length of the primordium. This is best seen in the larger primordia of similar length (e.g. L1 at day 2, L3 at day 8, and L4 at day 13), where the distal portions become more and more acute.

Up to day 6, only one, or barely one, leaf primordium has failed to overtop its apex; there were two such primordia on days 8 and 11; and three from day 13 onwards. This is an expression of the fact that there are longer intervals between the emergence of successive leaves than between the formation of successive primordia (see also Fig. 11), and that the height of the apex is itself increasing. Sharman (1947) and Cooper (1951) have shown that leaf primordia can accumulate in large numbers under the apices of some grasses, and it seems that even in wheat—which Sharman classifies as having a short type of apex—there is a tendency to accumulate leaf primordia in this way.

The actual volumes of the leaf primordia are presented in Table 4 and those for the associated corpus tissue are in Table 5. The former also appear on a logarithmic scale in the lower part of Figure 11. This figure attempts to integrate the information on volume change in the leaf primordia with the dry weight changes in the first four leaf blades of the primary shoot. Both dry weight and volume determinations were available for leaf 3 on day 8 and these values link the two sets of data. This link is admittedly rather tenuous, so it is reassuring to find the high degree of continuity shown by the data for leaves 1, 2, and 4. The value given for L5, day 21, is an estimated volume based on basal area and length; this also fits well into the general picture. The dry weight scale of Figure 11 has been extended down to give an idea of the total range of size (about five logarithmic cycles) traversed by the leaf from its first appearance as a recognizable primordium to full maturity.

Conversion of individual mean volumes to dry weights would involve the unwarranted assumption that the density of these meristematic tissues remained unchanged with time and between successive primordia. The data for leaf 3, day 8, imply a density of  $0.31 \text{ mg dry matter per mm}^3$ , a value which is very much higher than that from the data of Brown and Broadbent (1950) for pea root tips ( $0.075$  for the zone  $0.4\text{--}2.4 \text{ mm}$  from the tip). The higher value is too high to the extent of the shrinkage and compression suffered in preparation for volume estimation, but the difference is far too great to be explained on this basis. The mean cell size was very much smaller in the wheat apex (see below) than in the pea root tips, though the way that this could affect the issue is obscure.

It might be thought that the composite nature of the information presented in Figure 11 could invalidate any general conclusions which one might seek to draw from it. In the first place, the volumes represent the *whole* of the leaf primordia including the tunica-derived tissue at their junction with the stem. This tissue could *not* be included when the primordia were first dissected for dry weight determination

(e.g. leaf 3, day 8, in Plate 1 and in Fig. 11). However, the volume of the tunica-derived tissue was then a small fraction of the whole—only 1.4 per cent. in the case of leaf 3. Secondly, the ligule had not differentiated at the first dissection, and examination of many axes suggested that the whole of the portion removed would have developed into leaf blade and made little or no contribution to leaf sheath.

TABLE 4  
VOLUMES ( $\text{mm}^3 \times 10^{-3}$ ) OF LEAF PRIMORDIA (LAMINA + TUNICA)

Day	L1	L2	L3	L4	L5	L6	L7	L8	L9
1	110	10.4	1.5						
2	450	22.7	3.0						
3		74	6.7	0.9					
4		176	11.0	1.3					
6			40	4.9	0.8				
8			354	21.3	4.3	1.1			
11				89	12.8	2.9	1.0		
13				610	34.6	7.8	2.1	0.9	
15					62	12.6	3.2	1.4	0.7
18					276	36.7	9.7	2.8	1.3
21						195	38	5.5	2.2

For these reasons the dry weights of Figure 11 are for leaf blades only. However, the inclusion of leaf sheaths would have made only trifling differences to the time trends of Figure 11. Lastly, there is the implicit assumption that tissue density is

TABLE 5  
VOLUMES ( $\text{mm}^3 \times 10^{-3}$ ) OF CORPUS TISSUE ASSOCIATED WITH SUCCESSIVE LEAF PRIMORDIA

Day	L1	L2	L3	L4	L5	L6	L7	L8	L9
1	5.6	1.5	0.3						
2	6.6	1.8	0.3						
3		3.5	0.7	0.2					
4		5.8	1.5	0.2					
6			4.6	1.0	0.2				
8			14.3	4.0	0.9	0.3			
11				8.9	2.5	0.8	0.3		
13				21.8	5.9	1.9	0.8	0.4	
15					9.3	3.0	1.2	0.6	0.3
18					25.8	7.1	2.5	1.1	0.6
21						28.3	7.7	2.7	1.4

constant throughout the early development of the leaf primordia. Even this would not have serious consequences for the argument unless changes in tissue density were large and discontinuous. A little consideration will show that two- or even three-fold changes in density, provided they were continuous, would have little effect on the pattern of dry weight change implied by Figure 11.

The dry weight values of Figure 11 were established with precision and were therefore joined by continuous curves. Straight lines were fitted to the volume data for such time intervals as seemed justified by inspection. Leaves 1 and 2, and leaves 6-9 presented no difficulty here; terminal values for leaves 3 and 5 seemed to demand discontinuous change with time; and leaf 4 came in for rather special treatment because the dry weight extrapolation was deemed to be more reliable than the volume for day 13.

TABLE 6

RELATIVE GROWTH RATES FOR LEAVES (G/G/DAY) AND FOR LEAF PRIMORDIA (IN ITALICS, CM<sup>3</sup>/CM<sup>3</sup>/DAY) Bracketed values within harvest intervals do not differ significantly. For statistical treatment of the volume data see p. 419

	Harvest Interval (days)									
	1-2	2-3	3-4	4-6	6-8	8-11	11-13	13-15	15-18	18-21
L1	1.39		1.06	0.69	0.38	0.06	-0.02	0.02	0.01	-0.01
L2	0.78	1.14	0.88		1.07	0.51	0.17	0.08	0.01	0.00
L3	0.68	0.79	0.51	0.65	1.08	0.90	0.81	0.53	0.23	0.02
L4			0.40	0.67	0.74	0.48	0.93		0.82	0.38
L5					0.36	0.36	0.50	0.30	0.48	
L6					0.32	0.32	0.49	0.24	0.35	0.51
L7							0.40	0.21	0.36	0.41
L8								0.20	0.23	0.24
L9									0.21	—
S.E.	0.086	0.054	0.136	0.021	0.061	0.017	0.072	0.039	0.030	0.080
Linear regression†	***	*	**	n.s.	**	***	***	*	***	**
Non-linear regression†	*		n.s.		**	*	**	n.s.	*	n.s.

† Significant effects with probabilities less than 0.05, 0.01, and 0.001 are indicated by \*, \*\*, and \*\*\* respectively.

Table 6 summarizes the data of Figure 11 as relative growth rates, first for the leaf primordia (volume basis) and then for the early leaf blades (dry weight basis). The latter show the expected rapid falls from high values at the times of emergence to zero at days 11, 15, and 18 for leaves 1, 2, and 3 respectively. Unfortunately the time trends prior to emergence are not well established, being based on only four apices per occasion. In spite of this, it is possible to make rather precise comparisons of the relative growth rates for successive primordia within each harvest interval. This is because we are here concerned with relative volume changes between primordia within apices. The standard errors of Table 6 thus apply only within their respective time intervals, and beneath them are indicated the significances of the linear and non-linear regression coefficients of relative growth rate on leaf number. Detailed comment on these significances is scarcely necessary, but they support the general conclusions that follow. Up to interval 13-15 days the youngest



two or three primordia present at the time grow at similar rates, but the next oldest leaf primordium grows faster. After day 15, the younger primordia separate into two growth-rate groups, leaves 8 and 9 growing little more than half as fast as leaves 6 and 7. It may be significant that the spike primordium is initiated early in this same period, and that unpublished evidence suggests that a minimum of seven foliage leaves are formed on the primary shoot of this variety of wheat. It is even possible that the eighth and ninth primordia would have become foliar ridges of the lower part of the ear.

In the light of all the evidence of Figure 11, it also appears that successive leaves attain their maximum relative growth rates during the few days prior to their emergence from within the previous leaf. A further point is that there appears to be a discontinuity at about day 8 in the relative growth rates of the primordia. Thus the rates for leaves 3 and 4 and the beginning of leaf 5 are similar and average 0.66, whereas those for leaves 5 (later stage), 6, and 7 are also similar to one another and average 0.38. The exhaustion of seed reserves soon after day 8 (see Fig. 4) provides the most obvious explanation for the general reduction in relative rates of growth.

*(d) Cell-size Distribution within an Apex*

These data for an 11-day-old vegetative apex are presented in Figure 12. The mean cell volumes are plotted as a function of height above the base of the fourth primordium; the apex is the same as that used to illustrate the procedure for volume integration (Fig. 1) and the data are subject to the same errors due to shrinkage and compression as are the volume data already presented. The values for successive primordia overlap so completely that they are treated separately within Figure 12. To a limited extent the curves are repeated as broken lines to assist the reader to compare those for adjacent primordia or, in one case, the base of primordium 4 with corpus and tunica values.

Table 7 shows that the average cell volumes for the primordia present at day 11 are remarkably similar (ranging from 2.22 to 2.55 units for lamina plus tunica). The cells of the associated corpus tissue are larger but are also similar among themselves. Within the apical dome, however, the relative sizes of tunica and corpus cells becomes reversed, those nearest the tip being 3.0 units for the tunica, but only 2.4 units for the corpus (Fig. 12). The primordium of leaf 5 is the youngest to show a definite trend in cell size from base to tip, with a minimum of about 2.15 units near the base of the free part of the lamina. In the primordium of leaf 4, the region of minimal cell size is much more extensive, the actual minimum (about 2.25 units) being a third of the way up. A few cells near the tip of this primordium are quite large and may have entered the phase of cell enlargement. This primordium is just entering its phase of most rapid relative growth (Fig. 11).

The cell generations,  $n$ , of Table 7 are mean values based on the assumption that all cells had been dividing in all parts of the apex. The results must therefore be accepted with caution. The values for L7 indicate that about eight generations for the tunica, and six for the corpus, take place in the apex before a leaf primordium is recognizable. Since L4 was at this stage on day 3 it follows that it took seven more

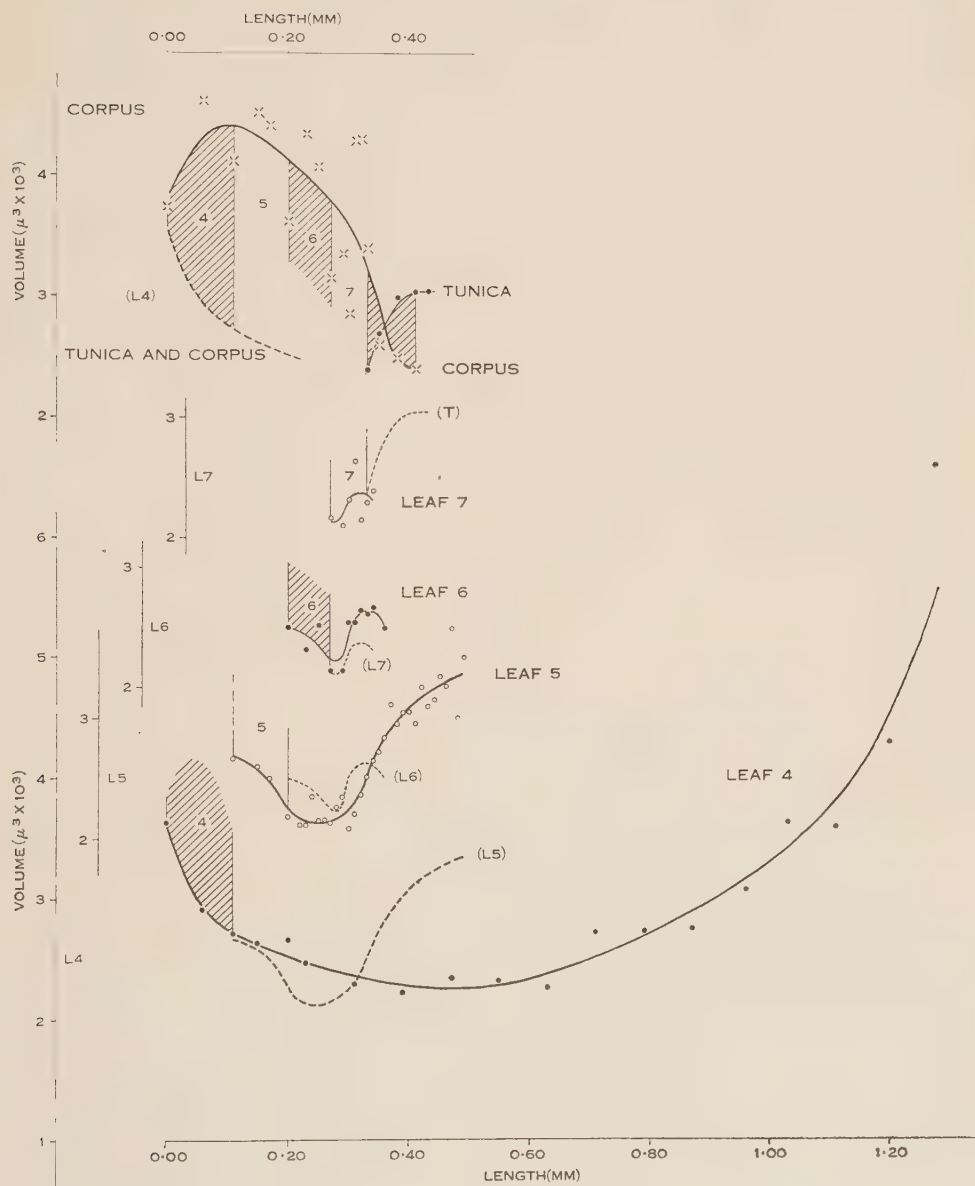


Fig. 12.—Mean cell-size distribution within an 11-day apex as a function of distance from the base of the fourth internode (see diagram of Fig. 1). The distributions for *L4*–*L7*, and for the tunica and corpus are shown separately, together with the limits of the appropriate internodes (shaded alternately).

generations and 8 days to acquire its cell number at day 11, which gives a mean generation time of 27 hr. If, as seems likely, the mean cell volumes were about the same for all leaf primordia prior to the onset of cell enlargement, the volume data of Table 4 indicate that the mean generation times ranged from about 12 hr for L1 to as much as 3 days for L8 and L9. The discrepancy of two generations between the values for tunica and corpus would seem to be explainable in terms of the geometry of the apex.

#### IV. DISCUSSION

The procedure of serial reconstruction has so far been used mainly for the description of form changes in embryos and embryonic parts of plants. Good examples

TABLE 7  
SUMMARY OF VOLUMES, NUMBERS OF CELLS, MEAN CELL VOLUMES, AND CELL GENERATIONS FOR  
THE APEX, LEAF PRIMORDIA, AND ASSOCIATED CORPUS TISSUE AT DAY 11

Attribute and Unit	Part*	Apex	L7	L6	L5	L4
Volume ( $\text{mm}^3 \times 10^{-3}$ )	L+T	0.92	0.85	2.75	13.7	100.5
	C	0.26	0.36	0.80	3.0	9.7
Number of cells	L+T	318	383	1195	5372	39,707
	C	94	98	195	741	2,407
Average cell volume ( $\mu^3 \times 10^3$ )	L+T	2.89	2.22	2.30	2.55	2.53
	C	2.77	3.67	4.10	4.05	4.05
Cell generations ( $n$ ), where cell No. = $2^n$	L+T	8.3	8.6	10.3	12.4	15.3
	C	6.6	6.6	7.6	9.5	11.2

\* L+T = lamina plus tunica; tunica only, for the apex. C = corpus or tissue derived from the corpus.

were provided by Avery (1930), McCall (1934), and Boyd and Avery (1936) for the developmental anatomy and morphology of wheat, oats, and maize embryos and seedlings. Avery (1933*a*, 1933*b*) presented similar studies of early development in the seedling and leaf primordia of tobacco. Randolph (1936) and Merry (1941) described the developmental morphology of the caryopsis and embryo of maize and barley respectively. More recently Jacobs and Morrow (1957) made use of serial reconstruction for the study of xylem development in the shoot apex of *Coleus*. Perhaps the most complete account of the developmental anatomy of the shoot of any plant, however, has been that of Sharman (1942) for maize. This work also attempted to relate the sequence of events in the development of the vascular system to the probable movement of food and water during growth. For the apical region of the shoot of *Lupinus albus*, Sunderland and Brown (1956) have devised new dissection techniques for the determination of the volumes and numbers of cells in young primordia and internodes. Protein contents and respiration rates within the same apical system have since been presented by Sunderland, Heyes, and Brown

(1957) and Sunderland (1960) has studied the contribution of cell division and expansion to the growth of leaves of *Lupinus albus* and *Helianthus annuus*.

The details of foliar histogenesis are now well understood for the Gramineae. Minor differences in the accounts of Rösler (1928) for wheat, Kliem (1937) for oats, and Sharman (1945) for *Agropyron* are discussed by Barnard (1955), and it is clear that foliage leaves arise as the result of periclinal divisions, usually restricted to the hypodermis and dermatogen just below the apex. These divisions spread laterally and soon give rise to a collar of tissue surrounding the axis (see Plate 3). By contrast the origin of the vegetative bud is by periclinal divisions of subhypodermal cells, the cells of the more superficial layers only dividing anticlinally. This difference of origin may be correlated with the nature of the leaf as an organ of limited growth, and of the bud as one of unlimited growth reduplicating the whole apex. To distinguish the superficial and deep-seated tissues in the present work, the terms "tunica" and "corpus" have been used (Fig. 1) for convenience rather than for theoretical reasons. However, as defined, they express the fact that the leaf primordia of wheat are the product of the two outer cell layers alone.

It is appropriate now to attempt an integrated picture of the early growth of the primary shoot of the wheat plant. Quite the best index of growth for this purpose is the relative growth rate,  $R$ , and this is adopted in the diagrams of Figures 13 and 14. These portray the relative rates of dry weight change in the main parts (including roots) and in successive individual leaves respectively. These rates are in fact based on the slopes of the appropriate curves of Figures 5 and 11.

Attention has been drawn to the probable influence of seed reserves on growth rates. The relative growth rates for leaves,  $R_L$ , and for roots,  $R_R$ , are exceptionally high prior to day 5, and even that for the stem,  $R_S$ , is a great deal higher than during the next five days. Another striking feature of Figure 13 is the contrast between the time trends for  $R_L$  and  $R_S$  such that  $R_S$  changes from being well below  $R_L$  to values which are greater than those for  $R_L$ . This reversal might be thought to be due to the inclusion of small tillers with the stems after day 15, but it has since been confirmed in an experiment in which the tillers were weighed separately. The reversal is an expression of the change in dominance from leaf growth to stem growth. With this goes the progressive increase in the rate of growth of the apex (see p. 416 and Figs. 9 and 10) and a general decrease in the  $R$  values for successive leaf primordia (Fig. 14). Even the corpus tissue associated with L1-L4 at first grows slowly or not at all (Table 5) at times when the primordia themselves are growing very fast (Table 4). By contrast, the lower diagrams of Figure 10 show for the end of the experiment that the apex, and with it the corpus tissue, was then growing faster than primordia 8 and 9. These trends reach their conclusion, as far as the primary shoot is concerned, at the double-ridge stage of inflorescence initiation, when further development of the lower ridge is suppressed, while the upper ridge develops as a spikelet. There is clearly a need to subject the developing inflorescence to the same quantitative description before attempting to carry this analysis further.

The pattern of development revealed by the  $R$  values for the leaves (Fig. 14) is a complex one, and interpretation of it is necessarily tentative. The most regular feature is that  $R$  for each of the early leaves rises to a maximum just prior to



emergence and then falls asymptotically to zero. Unpublished evidence indicates that  $R_{L5}-R_{L7}$  would also follow such a course, and that the maxima themselves would fall on a curve which was asymptotic to an  $R$  value between 0.5 and 0.6. The individual curves for post-maximal growth are those to be expected for organs

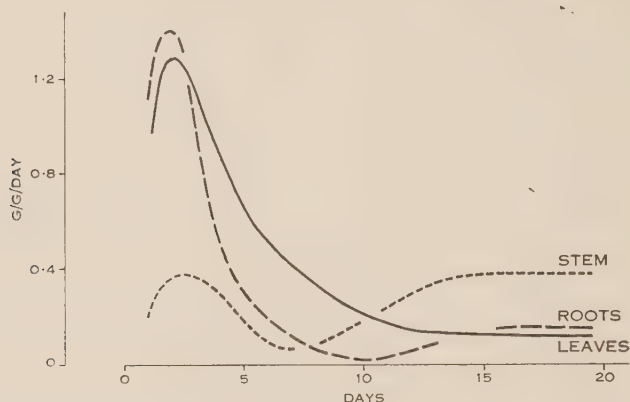


Fig. 13.—Relative growth rates for leaves, stem, and roots as a function of time.

passing through the phases of maturation and senescence. Since final leaf size increases with leaf number,  $R$  must be maintained at high values for a longer time with each successive leaf, the more so that its maximum is less rather than greater with each successive leaf. This is clearly true within the limits of the data.

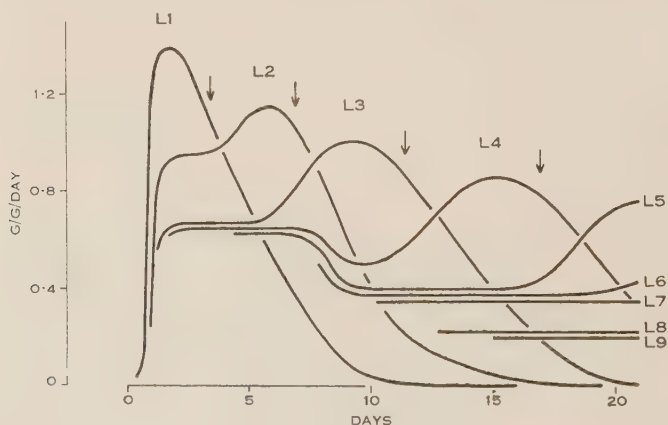


Fig. 14.—Relative growth rates for successive leaves of the primary shoot of wheat as a function of time. The arrows mark the times of emergence of the first four leaves.

Perhaps the most novel feature of the pattern of development revealed by Figure 14 is the variety of trend for  $R$  prior to leaf emergence. It is commonly supposed that the growth of an organ is exponential, or nearly so, for some time, and that it then falls away from exponentiality as the processes of maturation set in

Here, however, the leaf primordium tends to show an early phase of exponential growth followed by a substantial *increase* in the exponent to what may well prove to be a second, though briefer, phase of exponential growth. Plate 1 demonstrates, for the third leaf primordium, the visible consequences of this increase in the exponent, for the relative growth rate is clearly greater for the second 2-day period (cf. Fig. 11). If, characteristically, there are two distinct phases of exponential growth for leaf primordia, it is reasonable to suppose that the first phase for L1, and parts of those for L2 and L3 took place during embryo development.

An obvious basis for two distinct phases of exponential growth would exist if it could be shown that the cell division and cell expansion phases were fairly distinct for the leaf primordia of wheat. The evidence, as far as it goes, suggests that the onset of cell expansion may coincide with the increase in  $R$ , but it does not explain why the rate does increase. It has yet to be shown whether cell division continues for wheat in the manner shown by Sunderland (1960) for lupin and sunflower leaves. It is possible that the onset of cell expansion is conditioned by the timing of endogenous auxin production or by interactions with other growth regulators. However, there seems little direct evidence on this point. For the leaves of *Solidago sempervirens* L., Goodwin (1937*b*) showed that, with the cessation of cell division and the onset of cell enlargement, there was a sudden change in the length-breadth growth relations, and he suggests a correlation with the presence of large amounts of auxin demonstrated earlier (Goodwin 1937*a*). Another possibility is that effective vascular connection seems to be made at this time, for the first protophloem elements of the median vascular strand were then first seen to have been differentiated. By way of example, Plate 2 shows transverse sections of an 11-day seedling for which vascular differentiation was well advanced in L3 (just prior to emergence), but had scarcely begun in L4 (at beginning of rapid growth phase). The median strand has single protophloem and protoxylem elements, and two other strands have single protophloem elements only. According to Jacobs (1956), auxin is also implicated in xylem differentiation, and there is a clear case for a detailed study of the timing of effective vascular connection with the sources of substrate for growth. Prior to the establishment of such connections, all such substrate must get to the young primordia by active transport across a region of small, undifferentiated, and more or less isodiametric cells. Such conditions are consistent with the low rates observed for early primordium development.

Before leaving this question of exponential growth in leaf primordia, it is worth comparing the present results with those for *Lupinus albus* reported by Sunderland and Brown (1956). Table 4 and Figure 3 of their paper show that the volumes of successive primordia and their cell numbers increase exponentially with plastochron age. Accepting a plastochron interval of 2 days, this volume increase is equivalent to an  $R$  value of 0.32, which is somewhat less than that for L7 for the wheat seedling, though covering a similar range of absolute size (Fig. 11). As with L7, however, there would still be plenty of time for a later increase in  $R$  should such be characteristic for the later development of leaf primordia in general. A difference for *Lupinus* is that mean cell volume increases appreciably during what appears to be the early exponential phase of volume increase. The more recent study by Sunderland (1960) includes volume change in the fifth primordium of lupin and

in the second primordium of sunflower. In both cases there is an indication that  $R$  increases with time, but the fresh weight data which follow do not support this trend.

Another phenomenon which appears also to be correlated with the onset of the higher rate of primordial growth is a rather sudden increase in the stainability of the cytoplasm of the cells, particularly in the lower part of the primordium. This is exemplified in the lower figure of Plate 2, where L4 is stained more heavily than either L3 or L5. Only in the apex is the staining as heavy as in L4. The increase in stainability is likely to be due to an increase in ribonucleic acid and to imply an increase in the potential for protein synthesis. Bünning (1952, 1956) appears to be speaking of the same general phenomenon when he refers to an increased density of cytoplasm and an increase in the size of the nucleoli as characteristic of cells of high embryonality. He cites the outgrowth of leaf primordia as one among many examples of organ and tissue production which are conditioned by such increases in embryonality. According to Bünning, cells of high embryonality suppress the embryonality of neighbouring cells. This inhibiting effect is believed to be active for some distance within the tissues, and to be responsible for many processes of differentiation. The inhibiting effect is thought to be due to competition for specific substances rather than to the production of inhibitors.

Although Bünning was thinking of the initiation of leaf primordia rather than of their later development, it may be that the concept of intra-plant competition for specific substances applies with even greater force to the latter. Thus, it will be noted that each primordium enters its phase of maximal potential for growth just before emergence, and that from then on its growth shows a measure of independence from events elsewhere in the plant. Before this phase of maximal potential, and in spite of constant conditions for growth, the growth rates of the primordia seem to be determined by such events as the exhaustion of seed reserves and, later, the onset of inflorescence development. Initially the level of supply of energy substrate from seed reserves seems to have been sufficient to supply not only the heavy demand of L1 but also to maintain a higher rate in L2 than in L3 or L4. With the exhaustion of these reserves soon after day 8, the rate dropped to a lower level (L5-L7) presumably determined by products of photosynthesis in L1 and L2. Concerning the still lower rates for L8 and L9, one can only draw attention to the correlative rise in stem growth and the onset of spike development at about day 15.

This quantitative description of the growth of the primary shoot of the wheat plant shows that there is abundant scope for the operation of intra-plant competition for metabolites and nutrients. That assimilates appear to be the most likely substances competed for may simply follow from the fact that all mineral nutrients were maintained in adequate supply. In his studies of the influence of light and temperature on lateral bud development in ryegrass, however, Mitchell (1953a, 1953b) concluded that these factors operated mainly through their effects on the general level of energy substrate in the plant. Competition for the products of photosynthesis seems also to be a likely explanation for many of the compensatory phenomena discussed by Jacobs and Bullwinkel (1953) for *Coleus*. Still more recently, Milthorpe (1959) suggested that the rate of cell division in the terminal meristem of

cucumber was influenced mainly by the supply of assimilate. Events in the expanding bud were thought to be dominated first by competition for carbohydrate but later by competition for mineral substrates.

It will be evident that competition for substrate of any kind will be conditioned by the nearness of the competitive sinks to the source or sources of that substrate, by the efficiency of the transport systems involved, and by many other considerations

## V. ACKNOWLEDGMENTS

The author is greatly indebted to the late Mr. V. Petersons for technical and clerical assistance done faithfully and well. He is also indebted to Dr. C. Barnard for supervising the early stages of the histological studies and for helpful advice throughout the work; to Mr. G. A. McIntyre and Mr. G. N. Wilkinson, Division of Mathematical Statistics, C.S.I.R.O., for advice on statistical matters; to Dr. L. A. T. Ballard, Dr. L. T. Evans, and Dr. A. H. G. C. Rijven for constructive criticism of the manuscript, and to Miss T. C. van den Broek for technical assistance.

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## EXPLANATION OF PLATES 1–3

### PLATE 1

Early stages of growth of the third leaf primordium in wheat. The seedlings were harvested 4, 6, and 8 days after sowing.  $\times 42$ .

### PLATE 2

Fig. 1.—Transverse section of an 11-day wheat seedling. The inner leaf is *L4* section 87, of the same apex depicted in Figure 1. Most of *L3* is also visible and is in an advanced state of vascular differentiation.  $\times 165$ .

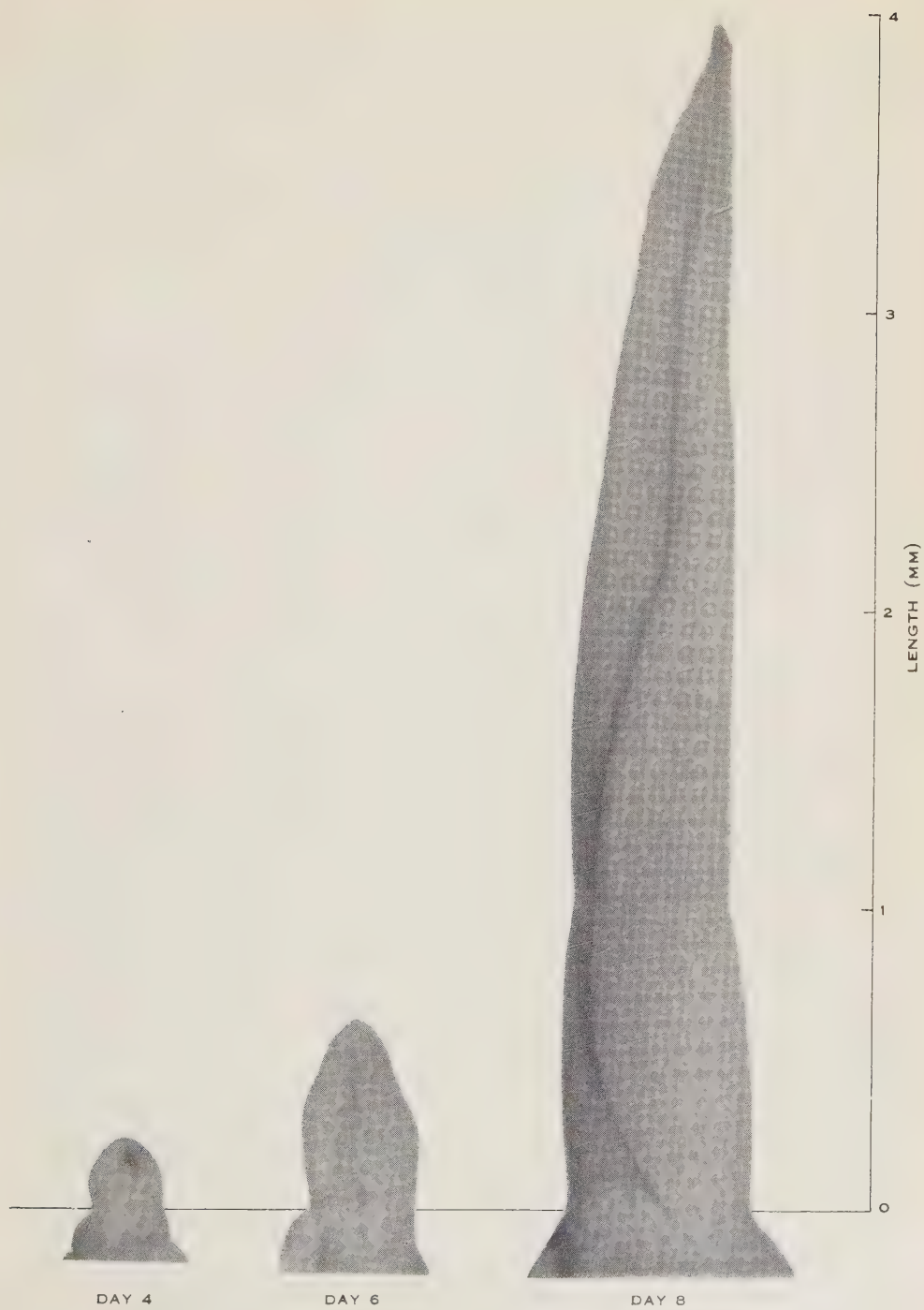
Fig. 2.—Transverse section of the same seedling (section 39, Fig. 1). At the centre is the growing point (heavily stained), *L5* (lightly stained), and then *L4* (heavily stained). *L3* is cut at the level of the ligule.  $\times 165$ .

### PLATE 3

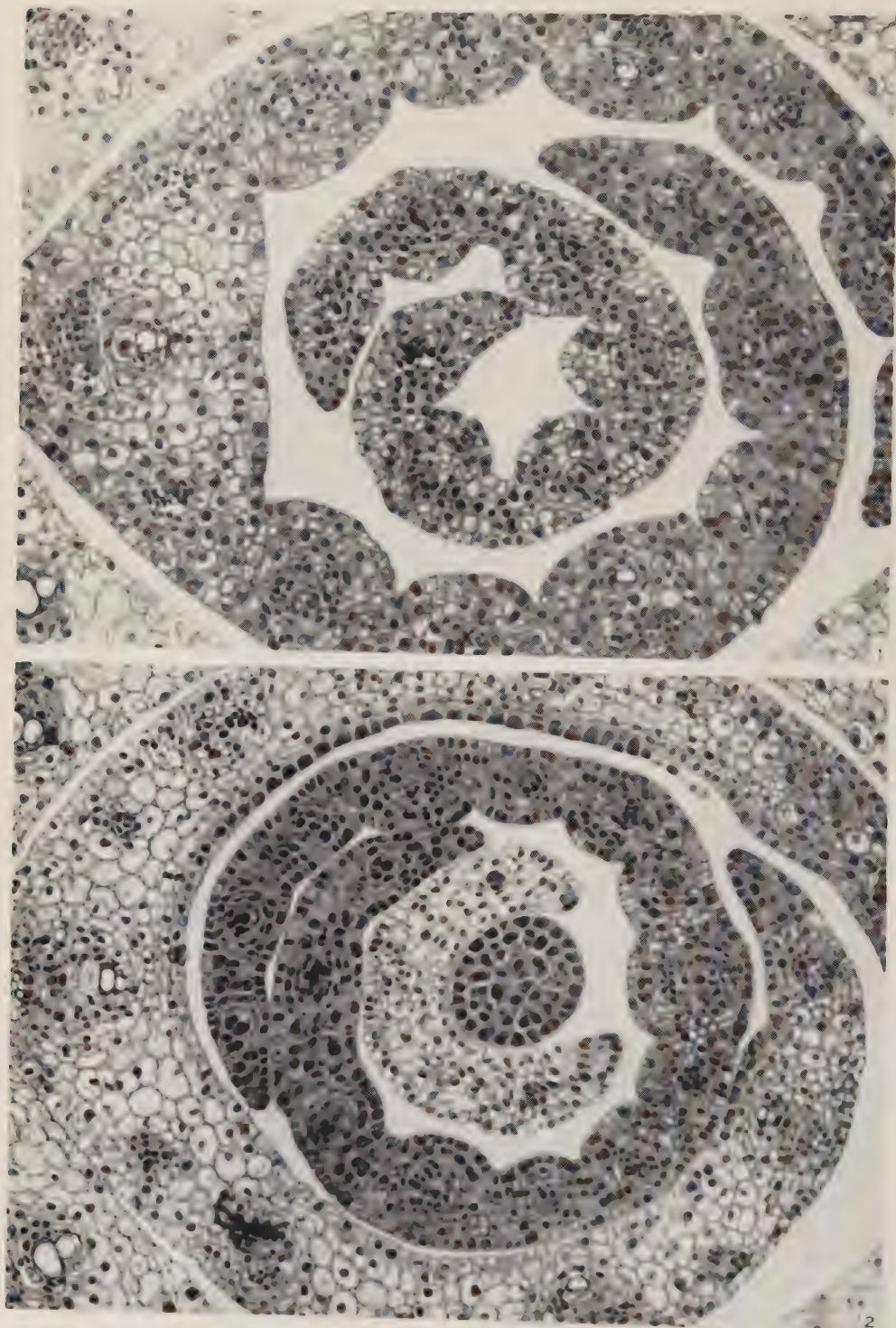
Fig. 1.—Longitudinal section of an 8-day wheat seedling, showing the two-layered tunica of the growing point. The overtopping leaf is *L4*; the smaller leaves may be identified from the appropriate diagram of Plate 3, Figure 2.  $\times 178$ .

Fig. 2.—Outline drawings of similar longitudinal sections for selected times throughout the experiment.  $\times 52$ .

## THE PHYSIOLOGY OF GROWTH IN THE WHEAT PLANT. I

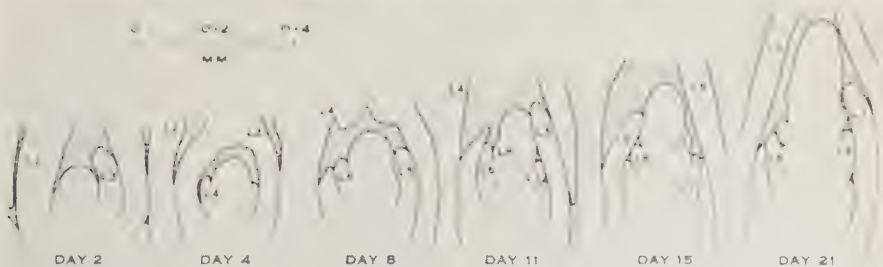
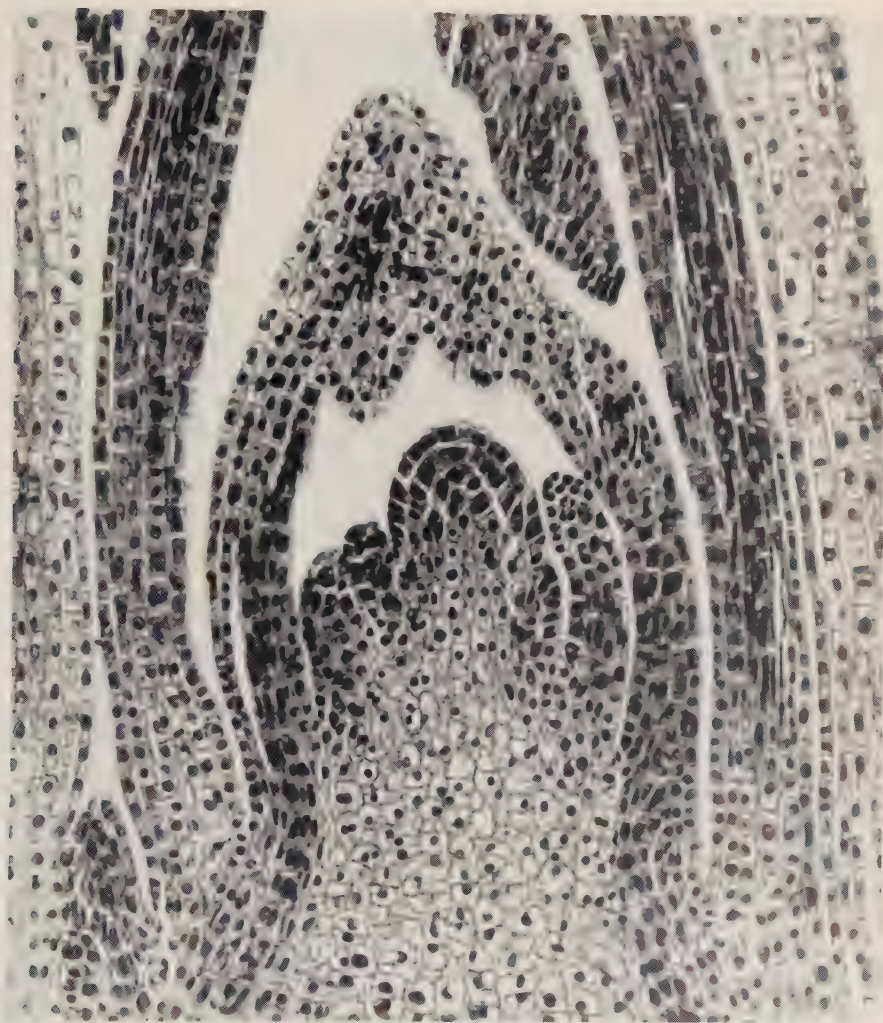


THE PHYSIOLOGY OF GROWTH IN THE WHEAT PLANT. I





THE PHYSIOLOGY OF GROWTH IN THE WHEAT PLANT. I







# INFLORESCENCE INITIATION IN *LOLIUM TEMULENTUM* L.

## II. EVIDENCE FOR INHIBITORY AND PROMOTIVE PHOTOPERIODIC PROCESSES INVOLVING TRANSMISSIBLE PRODUCTS

By L. T. EVANS\*

[*Manuscript received April 12, 1960*]

### *Summary*

Plants of *Lolium temulentum*, raised in short days, were given an inductive treatment by exposure of one leaf blade to a 32-hr period of continuous illumination. Then either the leaf exposed to this one long light period or varying areas of lower leaves which were simultaneously in short-day conditions were removed at intervals after the long-day exposure. The longer the long-day leaves remained on the plants, the greater was the proportion of plants which initiated inflorescences and the greater the rate of development of their inflorescences. This was so even when short-day leaves were present above the long-day ones. The longer the short-day leaves remained, and the greater their area, the lower was the proportion of plants which initiated inflorescences.

Low temperatures during the long-day exposure, and particularly during the period of low intensity illumination, prevented subsequent inflorescence initiation.

It is concluded (1) that the effect of time of removal of the leaf blades exposed to the long light period indicates the production by them of a transmissible substance which can initiate inflorescence development, provided the temperature during the long light period is above 10°C; (2) that the effect of time of removal of leaves exposed only to short days indicates the production by them of a transmissible inhibitor to inflorescence initiation which competes with the stimulus translocated from the long-day leaves.

### I. INTRODUCTION

The exact role of light in controlling the flowering of long-day plants remains an enigma. The requirement for a daily period of high intensity light may be simply to provide photosynthates (Liverman 1955), which play only a preparatory, albeit necessary, role in the photoperiodic reactions. Beyond this the requirement for a daily light period of more than a certain length may be merely a need for the absence of darkness. Thus Lang (1952), in his masterly survey of the physiology of flowering, concludes that the essential element in the photoperiodic response of long-day plants is the inhibitory effect of long dark periods, and that the formation of the floral stimulus is not dependent on light, provided this dark inhibition is absent. He concludes further "that the inhibiting action of darkness is localized entirely within the leaves" since no evidence has been presented for the production of a transmissible inhibitory material. Consequently, he supposes that the process which leads to the appearance of the floral stimulus occurs in the leaves, and that

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the dark inhibition is directed against the formation, rather than against the functioning of this floral stimulus.

In this paper evidence is presented of the occurrence of an inhibitory process in leaves of *Lolium temulentum* L. kept in short days, and of a stimulatory process in leaves exposed to a single long period of light. In the experiments to be described either the leaves exposed only to short days, or those exposed to the long light period, were removed at intervals. The effect on inflorescence initiation of their time of removal provides indirect evidence that both the inhibitory and the stimulatory products are translocated from the short-day and from the long-day leaves respectively. Thus, instead of the dark inhibitor preventing the formation of the transmissible flower-promoting substances in the leaves, as Lang (1952) supposes, it appears to operate against the functioning of the flower-promoting substances produced by the long-day leaves, at some site other than the leaves, and probably at the shoot apex during the production of the ultimate floral stimulus.

## II. EXPERIMENTAL METHODS

Most of the materials and conditions for the experiments reported here have already been described in the first paper of this series (Evans 1960a). All plants were grown for at least 5 weeks in 8-hr days until the sixth leaf was fully expanded. The treated plants were then exposed to one long period of continuous light, by extension of the 8-hr period in daylight with 16 hr of incandescent light of either 2 or 15 f.c. intensity at plant height. They therefore had 32 hr of continuous illumination, which will be referred to below as the long-day exposure. They were then returned to the standard short-day conditions (8-hr photoperiods, at 25°C/20°C) for 3 more weeks before dissection of the apices of the primary shoot. Plants were recorded as having initiated inflorescences when they had at least reached the double ridges stage of differentiation.

In some treatments the whole shoot was exposed to the long day, in others only the blade of the sixth leaf on the main shoot. In the latter treatments the other leaves were either wrapped in aluminium foil or else removed just before the end of the first daylight period, at 4.30 p.m. on the given long day. In this way plants with a single leaf blade exposed to a single long day could have a variable area of leaves simultaneously exposed to short-day conditions. Then, either the short-day or the long-day leaf blades were cut off at various intervals after the beginning of the long-day exposure.

In the larger experiments, the wrapping of the short-day leaves with aluminium foil took about 2 hr, in all cases being completed by 4.30 p.m. Care was taken to distribute the earliest-wrapped plants at random among the various treatments. Unwrapping, the following morning, was always completed within the first half hour of the daylight period. The efficiency of wrapping as a short-day treatment was checked in each experiment by having totally wrapped plants under the long-day conditions, these being compared with control plants maintained in short-day conditions throughout. All handling of plants during the dark periods, for example for the removal of leaves, was carried out in weak green light and trial exposures of plants to this light in no case elicited any flowering response.

## III. RESULTS

*(a) Evidence for the Movement of a Flower-promoting Stimulus from Long-day Leaves*

The evidence is presumptive, since it is based on the effect on inflorescence initiation of the time of removal of the one leaf blade on each plant which was exposed to a long day. These effects were examined in four experiments. In half of the treatments, leaves other than the long-day leaf were in short days during the long-day exposure, and were left on the plants until dissection. In the other treatments these leaves were removed just before exposure to the long day: thus, when the blades of leaf 6 were subsequently removed, only the sheath of leaf 6, and those parts of the younger leaves enclosed by it, were left on these plants. The results for one experiment are given in Figures 1(a) and 1(b).

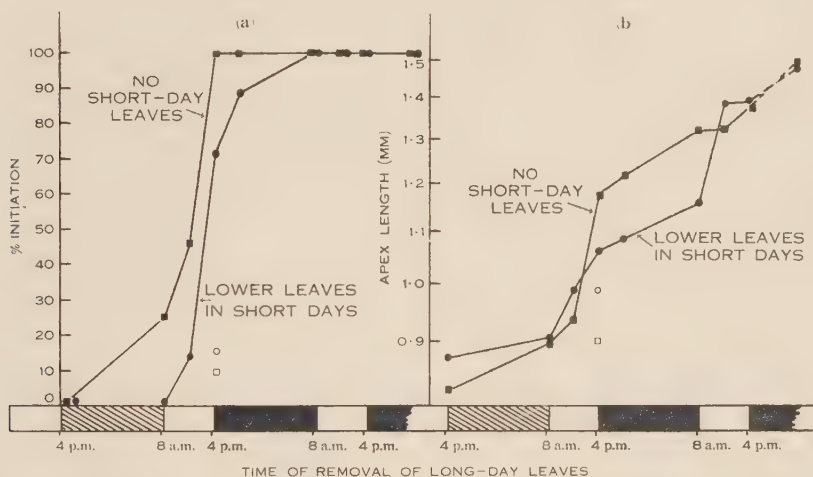


Fig. 1.—Effect of the time of removal of the blade of leaf 6 exposed to a single long day on the percentage of plants initiating inflorescences (a) and on the elongation of the shoot apices (b). Plants 48 days old at long-day treatment; supplementary illumination of 2 f.c. intensity; 8–12 plants per treatment. Plants, with short-day leaves (○) and without (□), given only low intensity illumination (2 f.c.) during the final 24 hr of the long light period.

Clearly, the longer the long-day leaf blades were left on the plant, the greater was the proportion of plants which initiated inflorescences (Fig. 1(a)) and the higher was the rate of apical development (Fig. 1(b)). It may be concluded that the curves indicate the translocation of a stimulus to inflorescence initiation out of the leaf blades exposed to the long light period.

For the plants with no short-day leaves it is apparent (from Fig. 1(a)) that removal of the long-day leaf blade at the end of the 16-hr period of low intensity light resulted in the subsequent initiation of inflorescences by one-quarter of the plants. Delaying the removal of the long-day leaf blade until the end of the following 8-hr period of daylight resulted in inflorescence initiation subsequently occurring in all plants. The results in Figure 1(b) indicate that still further delay in its removal resulted in increased rates of development of the initiated inflorescences.



Where short-day leaves were present on the plants during the long-day treatment, the pattern of results for both percentage flowering and apex length was similar to that where no short-day leaves were present. The longer the leaf blades exposed to the long light period were left on the plant, the greater was the proportion of plants subsequently initiating inflorescences, and the greater their rate of apical development. However, it is evident from Figure 1(a) that the presence of short-day

TABLE 1

EFFECT OF TIME OF REMOVAL, AND OF POSITION RELATIVE TO THE SHORT-DAY LEAVES, OF THE LEAF BLADE EXPOSED TO A SINGLE LONG LIGHT PERIOD, ON SUBSEQUENT PROGRESS TO FLOWERING. Plants 43 days old at long-day treatment; supplementary illumination of 15 f.c. intensity; 8-12 plants per treatment; plants dissected 3 weeks after long-day treatment. Mean areas of the blades of leaves 5 and 6 were 16.1 and 18.7 cm<sup>2</sup> respectively

Time of Removal of Long-day Leaf	Leaf 6 in Long Day, Leaf 5 in Short Day		Leaf 6 in Short Day, Leaf 5 in Long Day	
	Initiation (%)	Apex Length (mm)	Initiation (%)	Apex Length (mm)
4.30 p.m. (I) (short-day controls)	0	1.22	0	1.16
8.30 a.m. (II)	90	1.55	88	1.42
12.30 p.m. (II)	100	1.95	100	1.75
4.30 p.m. (II)	100	1.98	100	1.79
8.30 p.m. (II)	100	1.99	100	1.92
Leaf not removed (long-day controls)	100	2.55	100	2.23

leaves reduced the proportion of plants initiating inflorescences when the leaf blades exposed to the long light period were cut off during the second period of high intensity light.

In two treatments the plants, instead of being exposed to 8-hr of daylight of high intensity following the 16-hr period of low intensity illumination, were maintained under incandescent light of 2 f.c. intensity until the removal of the long-day leaves at the end of the long period of illumination. Under these conditions the proportion of plants initiating inflorescences, and the rate of their apical development, was much lower than in those plants defoliated at the same time after 8 hr of daylight (Figs. 1(a) and 1(b)).

The results of the second experiment were essentially similar to those described above, and differed only in the proportion of plants initiating inflorescences after the various times of leaf removal. There was no inflorescence initiation among plants from which the long-day leaf blades were cut off prior to the end of the long light period and only 70 per cent. and 50 per cent. among the plants without and

with short-day leaves respectively when defoliation occurred at the end of the long light period. Among the plants on which the long-day leaf blade was not removed, all those without short-day leaves, and 80 per cent. of those with short-day leaves subsequently initiated inflorescences.

In the third experiment, only 10 cm<sup>2</sup> of the sixth leaf blade was exposed to one long light period, and although this area is sufficient for inflorescence initiation in the absence of short-day leaves (Evans 1960a), it proved to be insufficient when all other leaves were in short days. Nevertheless, the length of the shoot apices increased significantly with the length of time before removal of the leaf blade exposed to a long light period.

In the fourth experiment, the effect of the time of removal of the leaf blade exposed to one long light period was compared in two series of treatments. One series was comparable to those described above in that leaf 6, the upper leaf, was given the long day, while leaf 5 was in short-day conditions. In the other series leaf 5 was given the long day while leaf 6 above it remained in short-day conditions. The results of the experiment are given in Table 1.

The results of both series are comparable to those described above in that the later the time of removal of the leaf blade exposed to the long light period the greater was the proportion of plants which had initiated inflorescences at the time of dissection and the greater the rate of development of their inflorescences. In the series in which the short-day leaf was above the long-day leaf the rate of inflorescence development was, however, slightly but consistently lower.

#### (b) *Evidence for the Movement of an Inhibitory Substance from Short-day Leaves*

From the results given in the preceding section it is evident that progress towards flowering in *L. temulentum* plants depends on the balance between the short-day and the long-day leaf areas. In the first experiment described above, the balance was such that all plants with the long-day leaves remaining on them could initiate inflorescences in spite of the presence of short-day leaves, while in the third, in which the leaf area exposed to the long light period was smaller, none did. In the experiments described below the leaf area exposed to the long light period was between these extremes, to permit expression of the effect of time of removal of the short-day leaves. The actual areas of the long-day leaves, the intensity of their illumination, and the areas of short-day leaves for the three experiments are given in Figure 2.

In all three experiments, the longer the short-day leaves remained on the plant after its exposure to a long light period, the smaller was the proportion of plants subsequently initiating inflorescences, and the slower their rate of apical development. The inhibition of initiation was greatest in the experiment with the highest ratio (6.5) of short-day leaf area to long-day leaf area, least in that with the lowest ratio (4.0). In all three experiments the inhibitory effect of the short-day leaf blades was almost maximal by the end of the succeeding 8-hr period of daylight. In two of the experiments a considerable inhibitory effect was apparent even when the short-day leaves were removed only 6 hr after the beginning of the dark period.

In a further experiment the area of leaf exposed to the long light period was kept constant, but both the area of the short-day leaves and the time at which they were cut off varied, with the results shown in Figures 3(a) and 3(b).

For all four times of removal of the short-day leaves, the greater their area the smaller was the proportion of plants which initiated inflorescences, and the slower their rate of apical development, with one minor exception. As in the preceding experiments, a marked inhibitory effect due to the presence of the short-day leaves was apparent even when they were removed only 6 hr after the beginning of the dark period.

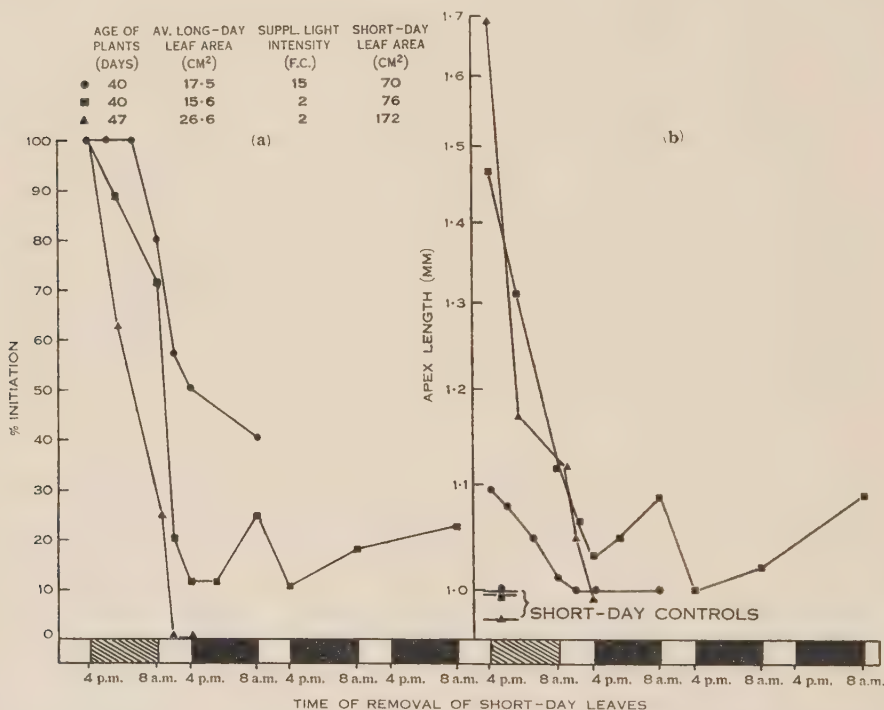


Fig. 2.—Effect of time of removal of the leaf blades kept in short-day conditions, during exposure of the sixth leaf to a single long light period, on the percentage of plants initiating inflorescences (a), and on the elongation of the shoot apices (b). 8–12 plants per treatment; other data given on figure.

### (c) Effect of Temperature during a Single Long-day Exposure

Four experiments have been carried out in which the temperature during the whole or part of a single long-day exposure, given to undefoliated plants of full photoperiodic sensitivity, has been varied.

In two experiments different temperatures were maintained throughout the first 24 hr of the long light period. The 8-hr periods of high intensity light in one were in daylight at temperatures 5°C above those of the following 16-hr periods, which were in incandescent light of 15 f.c. intensity. In the other, which involved

more extreme temperature treatments, the 8-hr period of high intensity light (1500 f.c.) was provided by fluorescent and incandescent lamps and the temperatures were held constant over the whole 24-hr period. For both these experiments dissections were carried out 5 and 35 days after the long-day exposures, allowing estimation of the relative growth rates of the inflorescences, with the results presented in Figure 4.

The marked influence of temperature on the inductive efficiency of a single long day is evident. Inflorescence initiation occurred in all plants of the treatments whose temperatures during the period of low intensity illumination ranged from 10 to 25°C, but there was a pronounced optimum at 25°C/20°C for the subsequent

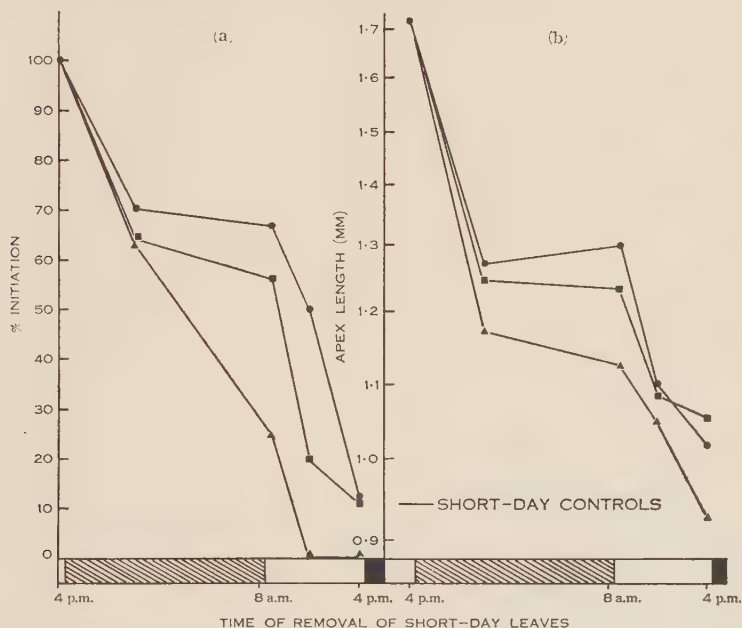


Fig. 3.—Effect of time of removal of the leaf blades kept in short-day conditions, during exposure of the sixth leaf to a single long day, on the percentage of plants initiating inflorescences (a) and on the elongation of the shoot apices (b). Plants 47 days old at long-day treatment; long-day leaf area 26.6 cm<sup>2</sup>; short-day leaf areas 21.1 cm<sup>2</sup> (leaf 5, ●); 45.9 cm<sup>2</sup> (leaves 3, 4, and 5, ■); or 172.4 cm<sup>2</sup> (all lower leaves, ▲).

growth rates of the initiated inflorescences and for the rate of their morphological development. Whereas the plants given a long day at 25°C/20°C had differentiated floret primordia at the time of the final dissection, those at 20°C/15°C and 30°C/25°C had only differentiated glume primordia and those at 15°C/10°C were only at the double ridges stage. At the highest temperature regime (30°C) only 75 per cent. of the plants initiated inflorescences, and at the lowest temperatures initiation was limited (12.5 per cent. at 7.5°C) or prevented (none at 3°C) despite the favourable photoperiod.



In the two remaining experiments the temperature during only part of the one long day was varied. In one experiment the temperature during the first 8-hr period of daylight was 25°C in all treatments while the temperature during the following 16-hr period of low intensity (15 f.c.) incandescent illumination varied from 4 to 35°C. In the other, the 16-hr period of low intensity illumination was at 20°C in all treatments, while the temperature during the preceding 8-hr period of daylight varied from 7.5 to 25°C. The results of the dissections made after all plants had spent a further 3 weeks in short days at 25°C/20°C are given in Figure 5.

Inflorescence initiation occurred in all plants whatever the temperature of the high intensity light period, but it is nevertheless evident that this temperature has had a pronounced after-effect on the rate of inflorescence development. The morphological differentiation of the induced apices also reflected this effect in so far

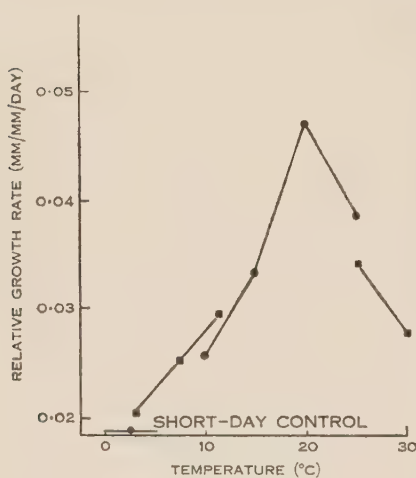


Fig. 4

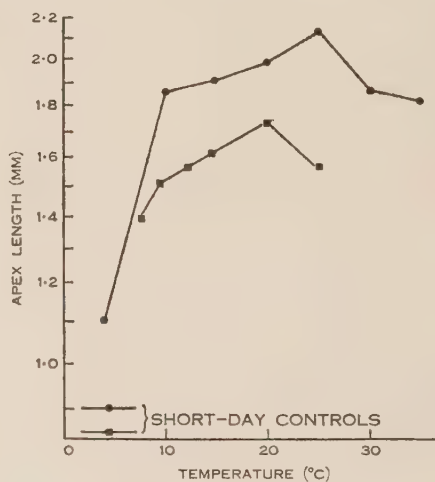


Fig. 5

Fig. 4.—Effect of temperature during a single day of continuous illumination on the subsequent relative growth rates of the shoot apices of plants of *L. temulentum*. ● Plants exposed to 8 hr of natural illumination, followed by 16 hr of incandescent light of 15 f.c. intensity at the temperatures indicated, which were 5°C lower than during the daylight period. ■ Plants exposed to 8 hr of high intensity (1500 f.c.) illumination from fluorescent and incandescent lamps, followed by 16 hr of incandescent light of 15 f.c. intensity at the same temperature.

Fig. 5.—Effect of temperature during part of a single day of continuous illumination on the mean apex length of plants subsequently returned to short days for 3 weeks. ● All treatments given 8 hr of daylight at 25°C, followed by 16 hr of incandescent illumination of 15 f.c. intensity at the temperatures indicated. ■ All treatments given 16 hr of incandescent illumination of 15 f.c. intensity at 20°C, preceded by 8 hr of daylight at the temperatures indicated.

as those in daylight at 7.5°C had only reached the earliest double ridges stage, and those at 9.5°C advanced double ridges, while the remainder had at least differentiated glume primordia. The temperature during the second part of the one long day, the 16-hr period and low intensity illumination, had a decisive after-effect on inflorescence development. No inflorescence initiation occurred when the temperature

during this period was 4°C. All temperatures from 10 to 35°C permitted initiation in all plants, the rate of apical development being highest at 25°C. At this temperature plants had differentiated floret primordia at the time of dissection, whereas only lemma primordia were differentiated at the other temperatures.

#### IV. DISCUSSION

##### (a) *Dark Inhibitory Process and the Transmissibility of its Product*

The results of the experiments in which the short-day leaves of plants were removed at intervals provide direct evidence of the net inhibitory effect of short-day leaves on inflorescence initiation in *L. temulentum*. They also provide circumstantial evidence of the transmissibility of this inhibitory effect, and of the time of its translocation from the leaf blade.

It could be argued that the inhibitory effect of the short-day leaves when left on the plants beyond a certain time is due to their acting as sinks for stimulatory substances from the long-day leaves, or because they generate a solute stream opposed to that from those leaves. Two lines of evidence refute these explanations. The first is that the inhibitory leaves were, with one specific exception, those inserted below the long-day leaves and, according to Lang (1952), could not act in this way. Secondly, were the short-day leaves to operate in either of the ways suggested, their inhibitory effect could scarcely precede, as it usually does, the apparent translocation of the stimulatory effect from the leaves exposed to the long light period.

Thus, we may conclude that the dark inhibitor is transmissible and that it acts at a distance from the short-day leaves. Although previously there has been no clear evidence for the transmissibility of the dark inhibitor, so that Lang (1952) was forced to conclude that the inhibiting action of darkness was localized entirely within short-day leaves, three earlier experiments have been suggestive of it. Lang (1941) obtained flower initiation in plants of *Hyoscyamus niger* kept in short days, provided they were defoliated continuously. The inhibitory action of the short-day leaves must therefore have extended over some distance from the leaves. Lang (1952) considered that this was due to the diversion from the axis tissues to the leaves of material necessary for the production of the floral stimulus. With spinach, Withrow, Withrow, and Biebel (1943) found that when plants with only one or three mature upper leaves were given 26 long days they flowered, whereas those also having their lower leaves in short days did not. As these investigators did not remove the leaves developing in short days above the long-day leaves, their results are inconclusive, since the young short-day leaves could have been acting as sinks for the products of the long-day leaves. That this might be so is indicated by the results given in Table 1, and by the results of Chailahjan (1946) with *Sinapis*. He found that when the short-day leaf was situated between the long-day leaf and the apex its inhibitory effect was far greater than when it was situated below the long-day leaf. However, incision of the stem for 5–8 cm below the insertion of the short-day leaf, when this was above the long-day leaf, greatly reduced the inhibitory effect, and Chailahjan concluded that this depended on the distance of the short-day leaf from the apical bud rather than on its disposition with respect to the long-day leaf.

(b) *Long-day Promotive Process and the Transmissibility of Its Product*

The results of the experiments in which single leaves of *L. temulentum* plants were exposed to a single long period of continuous illumination and were removed at intervals during and after this, indicate the formation and translocation from these leaves of a stimulus capable of initiating inflorescence development both in the presence and in the absence of leaves simultaneously in short-day conditions. Most of the translocation from the leaf blades exposed to the long light period appeared to take place in the second 8-hr period of high intensity light. In plants with no short-day leaves, sufficient stimulus had been translocated from the long-day leaf blades by the end of the period of low intensity illumination to initiate inflorescence development in a proportion of the plants. It may therefore be concluded that the second period of high intensity light is not required to complete the long-day process. Nevertheless, exposure of the plants to low intensity light following the 24 hr of continuous light resulted in far slower translocation of the stimulus from the long-day leaves, a result which is suggestive of passive translocation of the stimulus along with photosynthates. Carr (1957) has obtained comparable results with *Xanthium*, while Guttridge (1959) has presented evidence for the passive translocation with photosynthates of a growth-promoting, flower-inhibiting substance from long-day donor strawberry plants to short-day receptors.

It has been suggested (Barber 1959) that the long-day leaves promote flowering in long-day plants merely by acting as sinks for transmissible inhibitory substances from the short-day leaves. Known patterns for the distribution of assimilates (Belikov 1958; Thaine, Ovenden, and Turner 1959) hardly support this view.

It might also be suggested that the long-day leaves exert an apparently promotive effect by generating a solute stream opposed to that from the short-day leaves. The timing of their promotive effect in relation to the time of movement of the inhibitor from the short-day leaves does not conflict with this suggestion, but other evidence does so. In the first place, results similar to those presented in Figures 1(a) and 1(b) were also obtained from treatments in which the relative positions of the long- and the short-day leaves were reversed, i.e. in which the long-day leaf was below the short-day leaf and hence unlikely to generate a solute stream opposed to that carrying the inhibitory substance to the site of inflorescence initiation. Also, if the only effect of a long day is the suppression of the dark inhibitory process, it is difficult to account for the pronounced effect of temperature during that one long day on subsequent progress to flowering. In fact, the finding that a long-day exposure cannot initiate inflorescence development when the period of supplementary low intensity illumination was at a low temperature can only be explained by the assumption that a promotive process which is optimal at about 25°C, and severely retarded at 4°C, takes place in leaves during exposure to long-day conditions. Morley and Evans (1959) and Doorenbos and Wellensiek (1959) have also found that low temperatures during the exposure of plants of *Trifolium subterraneum* and *T. pratense* to long days prevented subsequent flower initiation.

Moreover, if a dark inhibitory process only was involved in the flowering of long day plants, as von Denffer (1950) suggests, since that process is known to be



greatly retarded at low temperatures (Lang 1952), flowering could be expected to occur in plants kept in short days with high day and low night temperatures. However, *L. temulentum* plants kept for 2 weeks in 8-hr photoperiods at 25°C/4°C showed no advance towards inflorescence initiation in subsequent short days at 25°C/20°C. It may be concluded then that a positive stimulus to flowering is formed in leaves of *L. temulentum* during exposure to long days, provided the temperature is not too low, and that this stimulus is subsequently translocated from these leaves, probably along with photosynthates.

### (c) *Interrelation of the Inhibitory and Promotive Processes*

Leaves in short days have been shown to have a net inhibitory effect on inflorescence initiation in *L. temulentum* which is transmissible, and not merely localized within the short-day leaves. Similarly, leaves in long days have been shown to produce a stimulus to inflorescence initiation which is also transmissible. We may conclude, therefore, that the dark inhibitor does not operate against the formation of the flower-promoting substance within short-day leaves, as Lang (1952) supposed, but against its functioning, after translocation from long-day leaves, presumably at the site of the potential inflorescence and in the process which finally leads to the initiation of inflorescence development. Since *L. temulentum* plants require exposure to progressively fewer long days as they become older (Evans 1960a), and in view of the competitiveness of the products transmitted from the short-day and the long-day leaves, it must be presumed that the dark inhibitor does not accumulate at the site of inflorescence initiation. The fact that interpolation of short days among a number of long days did not reduce the inductive effect of the long days (Evans 1960b) supports this conclusion. This absence of any accumulation of the dark inhibitor at the site of inflorescence initiation could account for Wellensiek's (1960) failure to find an inhibitory action of darkness in his experiments with several long-day plants.

The results given in this paper raise the possibility that light may play a direct and positive role in the initiation of flowering in *L. temulentum*. Since the net inhibitory effect of leaves exposed to long dark periods is not confined to those leaves, but acts at a distance from them in opposition to products from leaves not so exposed, light may not only operate to suppress the inhibitory dark process but may also accelerate the promotive processes occurring in long-day leaves. Two distinct photochemical processes, with different action spectra, may therefore be involved in the initiation of flowering in long-day plants. If so, much of the apparent diversity among long-day plants in their spectral requirements for flower initiation could be explained, since the balance between the inhibitory and promotive processes is likely to vary from species to species.

### V. ACKNOWLEDGMENTS

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# THE EFFECT OF NUTRIENT DEFICIENCIES ON THE HILL REACTION OF ISOLATED CHLOROPLASTS FROM TOMATO

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## Summary

Tomato plants were grown deficient in each known essential macro- and micronutrient (except chlorine) and the effect of each deficiency on the Hill reaction activity of chloroplasts isolated from these plants was studied.

All deficiencies, except that of iron, resulted in chloroplasts with reduced Hill reaction activity per unit chlorophyll. Among the micronutrients, deficiencies of manganese, copper, zinc, boron, or molybdenum caused impairment of activity in that decreasing order of severity.

In no case was the activity of chloroplasts from a deficient plant enhanced by the addition of the missing nutrient to the chloroplast suspension.

A comparison of the absorption spectra of suspensions of chloroplasts from healthy and deficient plants, and of 80 per cent. acetone extracts of these suspensions, revealed no shifts in the absorption peaks, and no gross change in the relative proportions of the major components. In some deficiencies a small but significant decrease was observed in the ratio of chlorophyll *a* to chlorophyll *b*.

Chloroplasts from healthy and deficient plants were assayed over a range of limiting light intensities up to saturation, and the effect of light intensity on the degree of impairment was recorded. In some cases, the degree of impairment due to deficiency was measured at 30 and 10°C with saturating light intensity.

Some tentative conclusions are drawn concerning the site of the impaired component of the Hill reaction associated with a number of the deficiencies.

## I. INTRODUCTION

Manganese deficiency causes a reduction in the rate of photosynthesis per unit amount of chlorophyll in a number of algae. This is true whether photosynthesis is measured as CO<sub>2</sub> fixed, O<sub>2</sub> evolved in presence of CO<sub>2</sub>, or O<sub>2</sub> evolved in the presence of an artificial oxidant such as a quinone (Pirson 1937; Pirson, Tichy, and Wilhelmi 1952; Arnon 1954; Eyster, Brown, and Tanner 1956). This evidence indicates that manganese is essential, either directly or indirectly, for the Hill reaction (the photolysis of water, and the evolution of oxygen accompanied by the reduction of an artificial oxidant). Kessler (1955) demonstrated that in the green alga *Ankistrodesmus* manganese deficiency causes no reduction in the rate of the initial photolytic reaction, but is involved rather in the evolution of O<sub>2</sub> from the products of photolysis.

Evidence concerning the role of manganese, and other nutrients, in the Hill reaction of higher plants is meagre. Gerretsen (1949) has shown that CO<sub>2</sub> assimilation per unit leaf area of oat leaves was reduced in manganese deficiency. He also found that breis of manganese-deficient leaves without any added oxidant formed less peroxides upon illumination than those of normal leaves (Gerretsen 1950). Mehler

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(1951) found that manganous ions stimulated oxygen uptake by isolated chloroplasts in the presence of catalase and ethanol. Manganous ions have been shown to stimulate  $\text{CO}_2$  fixation, but not the Hill reaction, of isolated chloroplasts from spinach (Allen *et al.* 1955).

Little is known about the effects of other nutrient deficiencies on the Hill reaction, especially in higher plants. Kessler (1955, 1957) has shown that phosphate deficiency results in lowered photosynthesis and photoreduction in intact *Ankistrodesmus*, whilst iron deficiency lowers the rate of photoreduction much more than photosynthesis.

The aim of the present work was to test whether manganese deficiency in a higher plant results in a reduction in the Hill reaction activity as measured in isolated chloroplasts. Having determined that this was the case, the effect of other nutrient deficiencies on the Hill reaction was also studied in order to test whether the effect of manganese deficiency is specific.

Since most of the work in this field to date has been carried out with intact algae, it has not been possible to decide whether the effects observed were due to direct or indirect participation in the Hill reaction of the nutrients concerned. By working with chloroplasts isolated from deficient tissues, rather than with intact cells, it was possible to attempt direct restoration of the Hill reaction *in vitro*.

## II. METHODS

### (a) *Water Culture Methods*

Tomato plants (*Lycopersicon esculentum* Mill., cv. Bonny Best) were grown in nutrient solutions in a glass-house in which the air temperature was controlled to approximately 25°C by day and to 20°C by night. For studies of micronutrient deficiencies, seedlings were germinated over distilled water and then transferred to nutrient solution of the composition described by Tsui (1948). For macronutrient deficiency studies seeds were germinated in vermiculite and transferred to nutrient solution of the composition described by Hoagland and Arnon (1950). Each water-culture vessel (3-l. "Pyrex" beakers) held 12 plants, and the nutrient solutions, which were aerated continuously, were not changed during the 2-3 weeks growing period.

Nutrient solutions deficient in molybdenum were prepared by the method of Gentry and Sherrington (1950). Solutions deficient in copper, zinc, and manganese were prepared by the method of Stout and Arnon (1939). Solutions deficient in boron were prepared by dissolving A.R. grade salts in water from a stainless steel still. Boron contamination was further reduced by lining the beakers with thin polythene bags. Iron-deficient solutions were prepared from A.R. grade salts dissolved in distilled water.

In order to obtain plants of a convenient size it was necessary to add a supplement of the missing nutrient routinely to certain deficient cultures. The iron-deficient cultures were supplemented with a final concentration of 0.143  $\mu\text{M}$  Fe. Similarly, 0.182  $\mu\text{M}$  B, 0.035 mM K, 0.01 mM Ca, 0.056 mM N, 0.0133 mM Mg, and 0.0133 mM P was added to the corresponding deficient cultures.

*(b) Preparation of Chloroplasts*

For each chloroplast preparation sufficient plants were harvested to yield 2–5 g fresh weight of leaves. All the leaves of each plant (excluding cotyledons) were used. In order to minimize day-to-day variation due to diurnal fluctuations in the Hill reaction activity (Hill and Scarisbrick 1939; A. W. Galston and J. Miller, personal communication) plants were harvested at approximately the same hour (9.00 a.m.) each day.

The procedure adopted for the preparation of chloroplasts followed closely that of Jagendorf and Evans (1957). The major departure from their method was the omission of polyvinylpyrrolidone from the initial grinding solution. Leaves were macerated in 60 ml of solution containing sucrose (0.30M), KCl (0.01M), potassium phosphate (0.05M, pH 8.5), and sodium ethylenediaminetetra-acetate (0.01M, pH 8.5). After removal of the cell debris by filtration through four layers of fine cloth, chloroplasts were sedimented by centrifugation at 1000 *g* for 10 min. The chloroplast pellet was suspended in 40 ml of a solution containing sucrose (0.30M), KCl (0.01M), and potassium phosphate (0.05M, pH 7.3) and again sedimented at 1000 *g* for 10 min. The pellet was resuspended in the latter solution. This chloroplast suspension was then used for the determination of Hill reaction rates and chlorophyll content.

Chloroplasts prepared in this way were examined under phase-contrast at  $\times 1600$  magnification. The chloroplasts appeared predominantly intact and undamaged, and were accompanied by insignificant amounts of other cell constituents excepting starch. The amount of starch in each preparation varied with the starch content of the leaf samples. Chlorophyll assays carried out on chloroplast preparations and on whole plants indicated that more than one-third of the total plant chlorophyll was contained in the chloroplast preparation.

Our aim in these experiments was to compare the activity of chloroplasts from deficient plants at an early stage of deficiency, with those from the appropriate healthy control plants. Comparisons between grossly deficient and healthy plants were avoided because of the extreme difference in size and morphology. Plants were harvested as soon as each deficiency was evidenced either by reduced fresh weight of whole tops, or by the commencement of characteristic deficiency symptoms. In most cases subsequent harvests were made within a period of 7 days.

*(c) Assay of Hill Reaction Rates*

These assays were carried out according to the procedure of Jagendorf and Evans (1957). This involves measuring the change in optical density (O.D.) at 620  $m\mu$  upon illumination for 45 sec of a reaction mixture containing chloroplasts, approximately 0.1M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.3, and 0.07  $\mu$ moles of *o*-chlorophenol-2,6-dichloroindophenol (B.D.H.) in a total volume of 3.0 ml. Illumination at more than saturating intensity was provided by a 250-W "Photoflood" lamp. The amount of chloroplasts ( $\equiv 2$ –10  $\mu$ g chlorophyll) in the reaction mixture was varied so as to give an O.D. change of 0.09–0.120 upon illumination for 45 sec. O.D. measurements were made in a Unicam SP 600 spectrophotometer and, unless otherwise noted, assays were carried out at 30°C.



Where a range of light intensities was required, the distance between the reaction vessel and the light source was varied. Light intensities were determined with an E.E.L. photoelectric illuminance meter.

Phosphate buffer used in the preparation of the chloroplasts and Tris buffer used in the Hill reaction assay were extracted with 8-hydroxyquinoline in chloroform in order to remove heavy metal contaminants.

Total chlorophyll concentration, and the relative proportions of chlorophyll *a* and *b* were calculated from the O.D. at 663  $m\mu$  and 645  $m\mu$  of an 80 per cent. acetone extract of the chloroplast suspension, as described by Arnon (1949). Hill reaction rates are expressed as the change in optical density at 620  $m\mu$  per 45 sec per

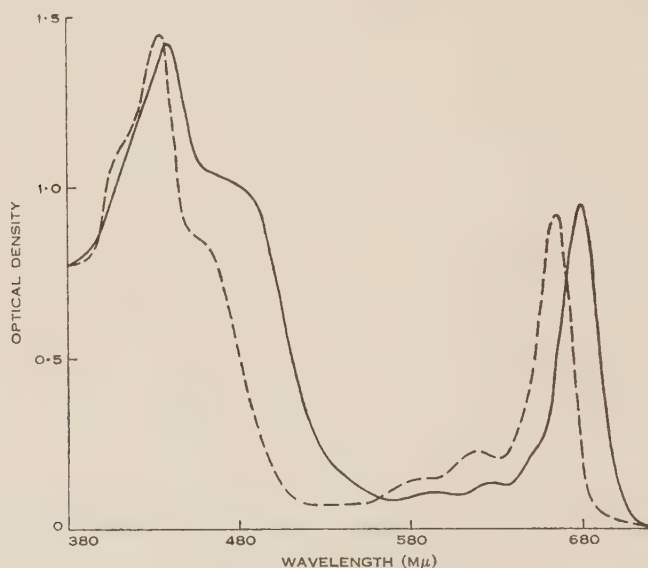


Fig. 1.—Absorption spectrum of isolated chloroplasts in sucrose-KCl-phosphate solution (—), and of an 80 per cent. acetone extract of isolated chloroplasts (-----).

mg chlorophyll. The expression of rates on the basis of chlorophyll present in the chloroplast preparation serves to eliminate the varying effects of the deficiencies on the chlorophyll status of the plants.

#### (d) *Spectral Examination of Chloroplast Pigments*

The absorption spectrum of normal and deficient chloroplasts was compared in two ways (1) by direct measurement on the chloroplast suspensions, and (2) by an examination of an 80 per cent. acetone extract of the chloroplast suspensions. In both cases measurements were made in an Optica CF4 spectrophotometer.

For the first method a strip of matt plastic tracing paper ("Astrafol", 0.01 in. in thickness) served as the standard against which the optical density of the chloroplast suspension was measured. This tracing paper had a constant optical density, relative to air, over the range from 380 to 710  $m\mu$ , and served as a reproducible

standard. The chloroplast suspensions prepared as described above were diluted with additional sucrose-phosphate-KCl solution until at 680  $m\mu$  the suspensions from both healthy and deficient plants had optical densities of approximately 1, relative to the "Astrafol" standard. The cuvettes containing the chloroplast suspensions were routinely placed at the back of the carriage-holder where they were as close as possible to the photocell.

Measurements of optical density were then made at 5- $m\mu$  intervals over the range from 380 to 710  $m\mu$ . A comparison with published spectra (Latimer 1959) and with the spectrum from the acetone extract of the chloroplast suspensions (Fig. 1) indicates that under these conditions in this instrument a representative portion of the scattered light is measured.

For the second method of comparison an 80 per cent. acetone extract of the chloroplast suspensions was prepared as described by Arnon (1949). Measurements were again made at 5- $m\mu$  intervals over the range from 380 to 710  $m\mu$ , using 80 per cent. acetone as the reference.

### III. RESULTS

#### (a) *Effect of Micronutrient Deficiencies*

The Hill reaction activity of chloroplasts from leaves of tomatoes deficient separately in each micronutrient was compared with that of chloroplasts from corresponding healthy control plants (Table 1). Deficiencies of manganese, copper, zinc, boron, or molybdenum resulted in impaired chloroplast activity per unit amount of chlorophyll in that decreasing order of severity. Iron deficiency was an exception since it consistently yielded chloroplasts which had slightly higher Hill reaction activity than chloroplasts from healthy plants when assayed at saturating light intensity. In one experiment boron deficiency did not cause marked reduction in activity at incipient deficiency.

The results in Table 1 were obtained from a number of experiments carried out over the course of 6 months (April to September, 1959). In some experiments successive harvests were made at 2- or 3-day intervals following the first detectable signs of deficiency. A considerable day-to-day variation was noted in the absolute Hill reaction rates of chloroplasts isolated from both healthy and deficient plants. Preparative and assay procedures contributed a small fraction of this variation. For example, four replicate preparations from uniform healthy plants harvested at the one time gave activities of 25.1, 22.3, 22.1, and 24.8 units. The major variation in rates appeared to arise from at least three sources: (1) a seasonal variation—in midwinter, especially if natural light was relatively poor, low activities were obtained; (2) a maturation effect—the activity of chloroplasts appeared to increase with increasing age of the plant up to about 3 weeks; (3) a diurnal fluctuation—this was brought to our attention by A. W. Galston and J. Miller (personal communication) and confirmed by us. Within the first few hours of daylight there is a rapid, almost twofold, rise in activity, followed by a decline throughout the remainder of the day. The extent and time scale of this diurnal fluctuation appears to be dependent on prevailing lighting conditions. This diurnal fluctuation probably accounts for most of the variation between harvests found in our experiments.



Because of this variation in absolute rates, the activity of chloroplasts from deficient plants is also expressed as a percentage of the activity of chloroplasts from the appropriate control plants (Table 1). It can be seen that on a percentage basis the effect of a deficiency on chloroplast activity is fairly constant under our experimental conditions. This suggests that the degree of fluctuation of Hill reaction activity is of the same order in chloroplasts from both deficient and healthy plants.

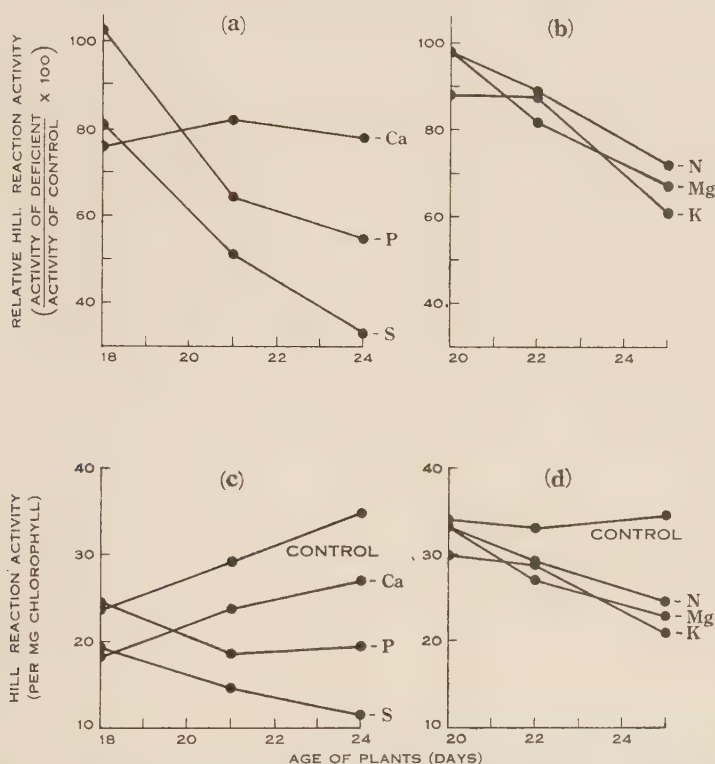


Fig. 2.—Effect of macronutrient deficiencies on Hill reaction activity of chloroplasts isolated from plants at intervals following the onset of deficiency: (c), (a), absolute activities (per mg chlorophyll) and relative activities (percentage of control value) respectively of chloroplasts isolated from plants deficient in calcium, phosphorus, or sulphur; (d), (b), absolute and relative activities respectively of chloroplasts isolated from plants deficient in nitrogen, magnesium, or potassium.

#### (b) Effect of Macronutrient Deficiencies

Figure 2 shows the results of two experiments in the first of which chloroplasts were prepared from plants deficient in either calcium, phosphorus, or sulphur (Figs. 2(a) and 2(c)). Three harvests were made in the 6-day period following the first detectable sign of deficiency. The results of a similar experiment in which three harvests were made of plants deficient in either nitrogen, magnesium, or potassium during the 5-day period following the first sign of deficiency are also shown in Figures 2(b) and 2(d).



A deficiency of any macronutrient resulted in a reduction, in some degree, of the Hill reaction activity per mg chlorophyll. It is not possible to compare the relative inhibitory effect of each deficiency in lowering the Hill reaction activity, because the plants are not necessarily under the same degree of nutritional stress. However, it is apparent from the trends in these two experiments that chloroplast activity, relative to that of control chloroplasts, declines rapidly at the onset of a deficiency of any one macronutrient (Figs. 2(a) and 2(b)). These trends were confirmed in other similar experiments. Deficiencies of sulphur and phosphorus led to the most rapid deterioration of activity under our experimental conditions.

TABLE 2  
EFFECT OF LIGHT INTENSITY OF THE ASSAY ON THE DEGREE OF IMPAIRMENT OF THE HILL REACTION  
DUE TO MICRONUTRIENT DEFICIENCIES  
Temperature = 20°C. Hill reaction activity expressed as ΔO.D. at 620 mμ/mg chlorophyll/45 sec

Light Intensity (f.c.)	$\frac{\text{Activity of "Deficient" Chloroplasts}}{\text{Activity of "Control" Chloroplasts}} \times 100$					
	—Mn	—Mo	—Fe	—B	—Zn	—Cu
2420	54	73	100	74	65	59
600	35	71	78	88	68	65
400	33	76	78	90	74	69
200	35	81	84	103	77	72
120	38	76	77	100	80	73

It is of interest that the effects of macronutrient deficiencies is accentuated markedly during the 5–6-day period covered by the harvests, whereas over this period the relative inhibition due to micronutrient deficiencies is fairly constant except in one case of boron deficiency. This suggests that from the visible onset of a deficiency the stress imposed by a deficiency of a macronutrient causes more rapid changes in metabolism than does a micronutrient deficiency.

(c) *Effect of Light Intensity of the Assay on the Degree of Impairment*

In order to determine whether the impairment caused by each deficiency was associated with a "light" or a "dark" step, chloroplasts from deficient and healthy plants were assayed for Hill reaction activity at a range of light intensities between 120 and 2420 f.c. At the lowest light intensity (120 f.c.) the activity of normal chloroplasts was reduced to about one-seventh of the saturation value. The activity of chloroplasts from deficient plants, relative to that of the appropriate control plants, is shown in Tables 2 and 3 for micronutrient and macronutrient

deficiencies respectively. It was found that chloroplasts from deficient plants fall into two categories, viz. (1) those in which the activity is impaired to the same, or even to a greater, extent at low light as at saturating intensities, and (2) those in which the degree of impairment is reduced at lower light intensities.

Among the micronutrients, manganese and molybdenum deficiencies resulted in chloroplasts in the first category whilst boron, zinc, or copper deficiency yielded chloroplasts which are in the second category (Table 2). Iron deficiency yielded chloroplasts which, although unimpaired at saturating intensities, consistently

TABLE 3

EFFECT OF LIGHT INTENSITY OF THE ASSAY ON THE DEGREE OF IMPAIRMENT OF THE HILL REACTION  
DUE TO MACRONUTRIENT DEFICIENCIES

Temperature = 20°C. Hill reaction activity expressed as  $\Delta$ O.D. at 620 m $\mu$ /mg chlorophyll/45 sec

Light Intensity (f.c.)	$\frac{\text{Activity of "Deficient" Chloroplasts}}{\text{Activity of "Control" Chloroplasts}} \times 100$					
	—S	—Mg	—N	—Ca	—K	—P
2420	54	60	79	73	73	58
600	45	62	71	74	79	60
400	42	63	67	75	86	64
200	45	67	67	72	94	75
120	—	67	66	74	102	81

showed inhibition at all limiting intensities. This set of experiments with micro-nutrient deficiencies was repeated at least four times for each deficiency, and, although the magnitude of the effects varied, the nature of the effect was consistent in each case.

Chloroplasts from plants deficient in sulphur, magnesium, nitrogen, or calcium showed approximately the same or increased impairment at low as at high light intensities, while with chloroplasts from potassium- or phosphorus-deficient plants the degree of impairment was reduced at low light (Table 3). However, in the case of chloroplasts from phosphorus-deficient and potassium-deficient plants the effect of light intensity was not consistent. In each case one out of four replicate experiments yielded chloroplasts whose behaviour with varying light intensity was the opposite of that shown in Table 3. It is possible that in these cases the state of the chloroplast alters with the stage of the deficiency.

The implications of these light-intensity experiments as to the impaired component of the Hill reaction associated with each deficiency will be considered in Section IV.

*(d) Effect of Temperature of the Assay on the Degree of Impairment*

The Hill reaction activity of chloroplasts from plants deficient in manganese, molybdenum, sulphur, calcium, magnesium, or nitrogen was tested at both 30 and 10°C with saturating light intensity. In all cases the percentage activity relative to that of control chloroplasts, was constant at both temperatures. Lowering the temperature from 30 to 10°C reduced the absolute activity of both control and deficient chloroplasts by approximately one-half.

TABLE 4

MEAN VALUES OF THE RATIO OF CHLOROPHYLL *a* TO CHLOROPHYLL *b* IN ISOLATED CHLOROPLASTS FROM HEALTHY AND DEFICIENT TOMATO PLANTS

Significant differences between control and deficient values indicated by asterisks

Deficiency	$\frac{\text{Chlorophyll } a}{\text{Chlorophyll } b}$		$\frac{\text{Chlorophyll } a}{\text{Chlorophylls } (a+b)} \times 100$	
	Control	Deficient	Control	Deficient
Iron	3.078	3.116	75.39	75.70
Copper	3.516	3.305*	77.86	76.77
Molybdenum	3.128	2.985**	75.78	74.91
Boron	3.064	2.914**	75.39	74.45
Manganese	3.192	2.823***	76.15	73.84
Zinc	3.087	2.914***	75.53	74.25
Magnesium	3.160	3.201	75.96	76.20
Calcium	3.149	3.225	75.90	76.33
Nitrogen	3.092	2.831**	75.56	73.90
Sulphur	3.222	2.901**	76.32	74.37
Phosphorus	3.240	2.636***	76.42	72.50
Potassium	3.189	2.722***	76.13	73.13

\* Difference significant at  $P < 0.05$ .

\*\* Difference significant at  $P < 0.01$ .

\*\*\* Difference significant at  $P < 0.001$ .

*(e) Attempted Restoration of Hill Reaction Activity in vitro*

For each deficiency which resulted in chloroplasts with reduced Hill reaction activity an attempt was made to restore the activity by the addition of the missing nutrient to the isolated chloroplast preparation. In no case was activity enhanced by this treatment. Macronutrients were added to the assay mixture at  $10^{-2}\text{M}$  final concentration, and micronutrients were tested from  $3 \times 10^{-6}$  to  $3 \times 10^{-4}\text{M}$  final concentration.

*(f) Effect of Nutrient Deficiencies on the Pigment Composition of Chloroplasts*

The results of a large number of assays of chloroplast suspensions showed that all deficiencies except iron, magnesium, and calcium caused a small but significant reduction in the ratio of chlorophyll *a* to chlorophyll *b* (Table 4). However, these

changes were small compared with the reduction in Hill reaction activity associated with the various deficiencies. Expression of activities on the basis of chlorophyll *a* alone did not substantially alter the percentage reduction in activity due to deficiency.

Direct spectrophotometric examination of intact chloroplasts isolated from healthy plants gave an absorption spectrum essentially similar to that described by Latimer (1959) for *Chlorella* (Fig. 1). Comparison of healthy and deficient chloroplasts gave no evidence of any shifts in the absorption maximum of chlorophyll *a*. Furthermore, no pronounced changes were found in the shorter wavelengths where at least part of the absorption is due to carotenes. An examination of the spectra of the 80 per cent. acetone extracts of chloroplasts from healthy and deficient plants also failed to reveal any gross changes (other than the chlorophyll *a*: chlorophyll *b* ratio) in the relative heights of the major absorption peaks.

#### IV. DISCUSSION

These results demonstrate that manganese deficiency in a higher plant, as in the case of algae (Eyster, Brown, and Tanner 1956), leads to a lowered Hill reaction rate per unit chlorophyll. On extending this investigation to other nutrient deficiencies it was found that this effect is by no means specific. A deficiency of any single macronutrient, or of any micronutrient other than iron, in some way induces changes which result in chloroplasts with reduced Hill reaction activity per unit of chlorophyll (Table 1; Fig. 2).

A reduction of Hill reaction rates as a result of a nutrient deficiency could be brought about in one or more of three ways. The deficient nutrient could be required as such directly in the Hill reaction, it could be a part of a more complex component of this reaction, or it could be essential in some reaction which in turn affects the formation of a Hill reaction component. In the last instance, the site of action of the nutrient could be outside the chloroplast. Since in no instance were impaired chloroplasts reactivated by the addition of the deficient nutrient *in vitro*, it can be concluded that the lowered activities observed were not due to lack of the deficient nutrient ion as such in the Hill reaction. If these are involved in this reaction they are present in adequate amounts even in washed chloroplasts from deficient plants. Photochemical reactions which respond to added manganous ions, such as those described by Gerretsen (1950) and by Mehler (1951), do not appear to be involved in the Hill reaction as measured by dye reduction.

Some of the effects which we have observed could be due to the association of natural inhibitors of the Hill reaction with certain deficiencies. Clendenning (1957) has reviewed evidence that naturally occurring compounds such as tannins can irreversibly inactivate chloroplasts during isolation. Cell sap of low pH is another potential source of inactivation. It is possible that both these factors could be affected by a nutrient deficiency and be responsible for lowered activities in isolated chloroplasts. Since the chloroplasts underwent a high dilution during isolation it is unlikely that any of the observed effects of deficiencies are due to reversible natural inhibitors. Consistent with this, when chloroplasts from plants deficient in any of the micronutrients were combined with normal chloroplasts their activities were additive.



By expressing Hill reaction activities on the basis of chlorophyll present in the chloroplast preparations we have sought to eliminate the effects of the various deficiencies on the formation of chlorophyll itself. Thus sulphur and iron deficiencies, although both characteristically resulting in reduced chlorophyll status, have markedly different effects on the Hill reaction rate per unit of chlorophyll. Sulphur deficiency apparently causes more drastic reduction in some other component of the Hill reaction than in chlorophyll, whilst iron deficiency does not (Fig. 2; Table 1). Comparison of activities on this basis implies that such chlorophyll as is formed in deficient plants is strictly comparable with that in healthy plants. The presence of abnormal, photochemically less active chlorophyll in deficient plants could result in an apparent reduction in other components of the Hill reaction. Any abnormality of the chlorophyll or its associated protein might be expected to result in a change in the characteristic absorption spectrum. However, examination of both the chloroplast suspensions and the 80 per cent. acetone extracts of these suspensions revealed no detectable qualitative difference between chloroplasts from healthy plants and from plants deficient in any nutrient. Although a small quantitative reduction was found in the proportion of chlorophyll *a* in the total chlorophyll under a number of deficiencies (Table 4), this was too slight to account for the reduced Hill reaction activities in terms of chlorophyll *a* as the only functional chlorophyll fraction. The differences in Hill reaction activity found in these experiments therefore do not appear to be explicable in terms of a grossly abnormal pigment complement resulting from nutrient deficiency. It is possible, however, that changes more subtle than those we have measured occur in both chloroplast composition and structure in deficient plants. Such changes could lead to reduced photochemical activity.

Bogorad *et al.* (1959) have reported that iron-deficient *Xanthium* leaves contain chloroplasts which have a granular disorganized appearance instead of the normal, highly organized lamellar structure of chloroplasts from healthy plants. However, the present results have shown that the chloroplast fraction of iron-deficient plants has a Hill reaction activity per unit of chlorophyll, at saturating light intensity, comparable with that of normal plants. It appears that highly organized chloroplast structure is not a prerequisite for Hill reaction activity, since small fragments of chloroplasts and even digitonin extracts of chloroplasts are known to retain this activity (Eversole and Wolken 1958).

The detailed mechanism of the steps which make up the Hill reaction is quite unknown. However, the overall reaction is known to consist of three components, viz. (1) the "light" step involving photochemical reactions through which water is split and an oxidized and reduced product are formed; (2) the "dark", enzymatic steps of the hydroxyl sequence which terminates in the liberation of molecular oxygen; and (3) the "dark", enzymatic steps in the reducing sequence whereby the reducing power is transferred to the added artificial oxidant—indophenol dye, (see diagram, p. 454). A decrease in the rate of any one of these reactions could result in a decreased overall Hill reaction rate (Brugger and Franck 1958). In order to obtain evidence as to which of these components is impaired by each deficiency, the effect of light intensity on the degree of impairment was studied. As a generalization, inhibitors of photosynthesis whose effects are lessened at low light intensities are

said to affect dark reactions, while those which are equally inhibitory at both high and low light intensities affect photochemical reactions (Rabinowitch 1945). However, the work of Gaffron and others on the effect of inhibitors on photoreducing algae demonstrates that this concept must be modified. Four inhibitors of photosynthesis, hydroxylamine (Weller and Franck 1941; Gaffron 1942), *o*-phenanthroline, phthiocol (Gaffron 1945*a*, 1945*b*), and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Bishop 1958) have been shown to act on the dark reactions of the oxygen evolution sequence. This is indicated by the fact that, at certain concentrations of these compounds, photosynthesis is inhibited while photoreduction is unaffected. In all cases photosynthesis is inhibited at both high and low light intensity. These examples prompt the generalization (Brugger and Franck 1958) that any factor which impairs the oxygen evolution sequence will, like an inhibitor of the photochemical step, result in inhibition of photosynthesis at all light intensities. It follows that, in the Hill reaction, inhibition which is dependent on light intensity will occur only when a dark reaction in the reductive sequence to indophenol dye is affected.

On this basis the present results (Tables 2 and 3) suggest that deficiencies of zinc, copper, or boron and probably postassium and phosphorus caused the impairment of some dark reaction in the reducing sequence to the indophenol dye, since in these cases the degree of impairment was reduced at low light intensities. On the other hand, with chloroplasts from plants deficient in either manganese, molybdenum, nitrogen, sulphur, calcium, or magnesium the impairment was as great, or greater, at low light intensities. This suggests that in these deficiencies either a light step, or a dark step involved in oxygen evolution has been affected. Chloroplasts from iron-deficient plants should probably be included in the latter category since, in this case, a reduction in Hill reaction activity was apparent only at limiting light intensities. This is consistent with the suggestion (Hill and Davenport 1952; Kamen 1956) that cytochrome *f* functions as an intermediate in the oxygen evolution sequence.

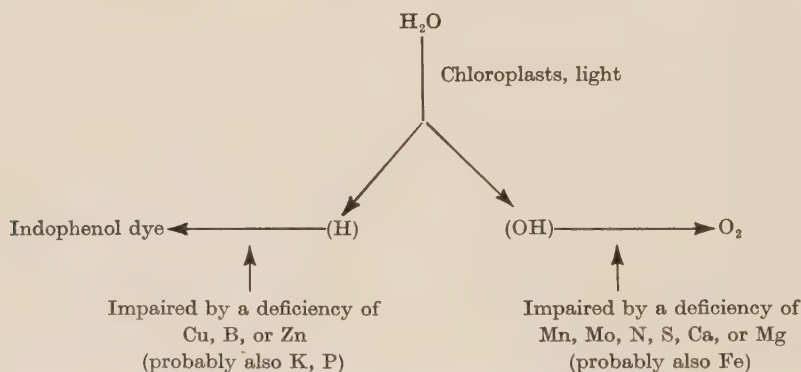
It should be possible to discriminate between impaired photochemical and impaired enzymatic reactions by virtue of their widely different temperature coefficients. Thus the  $Q_{10}$  of an enzymatic reaction is generally between 1 and 2, whereas the rate of a photochemical reaction is unaffected by temperature.

Under saturating light intensity, a decrease in temperature would result in a decrease in the Hill activity of the control chloroplasts. If an impaired enzymatic reaction is responsible for the reduced overall activity, a decrease in temperature would result in a decreased Hill activity. Since the rates of the control and enzymatically impaired chloroplasts are both reduced, no significant change in the relative rates would be observed by increasing the temperature. If a light step were impaired, on the other hand, a decrease in temperature would have no effect on the rate of the impaired reaction. Therefore a reduction in the relative degree of impairment should be observed by lowering the temperature.

The activity of those chloroplasts which showed undiminished degree of impairment at low light intensities was compared with that of normal chloroplasts at 30 and 10°C at saturating light intensity. (In the control chloroplasts the rate of the Hill reaction at 10°C was approximately one-half the rate at 30°C.) In no case was the degree of impairment affected by the change in temperature. This infers

that these deficiencies (manganese, molybdenum, nitrogen, sulphur, calcium, or magnesium) caused a reduction in the rate of some enzymatic reaction in the oxygen evolution sequence.

These conclusions concerning the site within the Hill reaction of the impairment caused by each deficiency can only be tentative. They are based on the assumption that each nutrient deficiency affects a single part of the Hill reaction. This is unlikely to be the case, especially for the macronutrients whose functions are manifold. Where a deficiency affects more than one site in the Hill reaction, only that site which is most impaired will be detected. With some nutrients the effect may vary with the degree of deficiency. However, the conclusions serve as a useful starting point for more detailed work on the effect of these nutrient deficiencies on the Hill reaction. They may be summarized as follows:



With respect to manganese deficiency these results are in accord with those found in studies on algae. Pirson, Tichy, and Wilhelmi (1952) and Arnon (1954) showed that the inhibition of photosynthesis caused by manganese deficiency is independent of light intensity. Working with cultures of the photoreducing algae *Ankistrodesmus*, Kessler (1955, 1957) demonstrated that manganese deficiency reduced photosynthesis but not photoreduction, and concluded that manganese was important in a dark reaction early in the hydroxyl sequence. Our results to date do not permit any conclusions as to the relative position in the hydroxyl sequence of the impairment caused by manganese deficiency in higher plants, but it is hoped to elucidate this in experiments now in progress.

It should be emphasized that the mere demonstration of a reduced metabolic activity associated with a nutrient deficiency in no way implies a direct function of this particular nutrient in that activity (cf. Pirson 1958). The effects observed could arise as an indirect consequence of the nutrient's direct action. It is felt that, to date, there is no unequivocal evidence of *direct* participation of any nutrient in the Hill reaction, although such a function has been claimed for manganese (Kessler 1957). With intact cells, as used by Kessler, the possibility of indirect action cannot be excluded, and in the present experiments no reactivation was found upon addition of manganous salts to isolated chloroplasts from manganese-deficient plants. The possibility remains, of course, that manganese participates in the Hill reaction in a complex molecule, such as a mangano-protein, which cannot be formed in isolated



chloroplasts. Indeed, the rapid restoration of photosynthesis upon addition of manganous salts to a manganese-deficient culture of *Scenedesmus* (Arnon 1954), and the increased manganese requirement of *Chlorella* under autotrophic as compared with heterotrophic conditions (Pirson and Bergmann 1955; Eyster *et al.* 1958) both suggest that manganese does participate directly in some photosynthetic reactions.

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# THE SYMBIOTIC SYNTHESIS OF AUXIN BY LEGUMES AND NODULE BACTERIA AND ITS ROLE IN NODULE DEVELOPMENT

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## Summary

When subterranean clover (*Trifolium subterraneum* L.) plants were grown for 3 weeks over distilled water, tryptophan could be detected in the root medium of both sterile cultures and those inoculated with *Rhizobium trifolii* 3 days earlier. Auxin could be detected only in the inoculated medium. The auxin had the chromatographic and growth properties of indole-3-acetic acid (IAA). Since nodule bacteria produce auxin only in the presence of tryptophan, which is a probable precursor of IAA, it is suggested that the tryptophan exuded by clover roots is converted to IAA by nodule bacteria. Auxin was still produced in the root medium when strains of *Rhizobium* which do not nodulate subterranean clover roots were used as inoculant, or when nitrate, which delays nodulation, was present in the medium.

Tryptophan, at high concentrations, delayed nodule initiation in lucerne (*Medicago sativa* L.) plants grown on a mineral salts agar, while  $\alpha$ -naphthaleneacetic acid, an auxin, also delayed initiation, and in addition decreased the total number of nodules formed and prevented many plants from forming nodules. An antiauxin, *p*-chlorophenoxyisobutyric acid, did not influence nodule initiation, but increased the rate of nodulation and the total number of nodules formed per plant. A root growth promotor,  $\alpha$ -(1-naphthylmethylsulphide)-propionic acid did not influence nodule initiation or number. Kinetin inhibited root growth, prevented some plants from nodulating, and reduced the number of nodules formed. Gibberellic acid slightly delayed nodule initiation, but greatly reduced nodule number, while root weight and nodule volume per plant were unchanged. Coconut milk inhibited nodule initiation.

A possible mechanism of root-hair infection and nodule inception is discussed.

## I. INTRODUCTION

The detection of an auxin in the nodules of a number of legumes led Thimann (1936, 1939) to postulate a role for auxin in nodule formation and growth. These and all subsequent detections of auxin in legume-nodule bacteria associations were made after the infection process and nodule initiation had occurred (see Pate 1958). In the present investigation the formation of auxin in legume root media (meaning here the media in which legume roots grew), at the time of infection and before nodule initiation, is studied. Also the effects of the presence in legume root media of auxin, auxin precursor, and other growth regulators upon nodule formation are studied.

## II. MATERIALS AND METHODS

### (a) Growth of Test Legumes

All plants were grown under bacteriologically controlled conditions.

(i) *For the Production of Tryptophan and Auxin.*—Seeds of subterranean clover (*Trifolium subterraneum* L., Mount Barker strain) were surface-sterilized and sown

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in 6 by 1 in. glass tubes (five seeds per tube) on moistened pads of glass wool over 10 ml of glass-distilled water or, when required, potassium nitrate solution. Since plants were grown for 21 days only and grew slowly, seed reserves provided ample nutrient supply. After sowing, tubes were held at 4°C for 48 hr to break seed dormancy, then at 26°C in darkness for 48 hr to give maximal germination. After germination they were placed in a louvre-shaded glass-house (Hely 1959). Eighteen days after sowing, the liquid medium was inoculated where necessary. Three days later the seedlings were removed from the tubes and the root medium was collected.

(ii) *For Nodulation Studies*.—Seeds of lucerne (*Medicago sativa* L., Hunter River strain) or barrel medic (*Medicago tribuloides* Desr., strain 173) were surface-sterilized and sown on mineral agar slopes in 6 by  $\frac{3}{4}$  in. test tubes using a technique which was essentially that of Thornton (1930). These species were used in nodulation studies because they nodulate more slowly than subterranean clover.

Seeds were germinated under the same conditions as the subterranean clover above, before being placed in the controlled conditions of 12 hr of light at 25°C and 12 hr of darkness at 20°C. Light was provided by fluorescent tubes supplemented with incandescent bulbs; the average intensity outside the seedling-growth tubes was 900 f.c. Four days after sowing, inoculum and, where necessary, 1 ml of solutions of tryptophan, potassium nitrate, a plant growth regulator, or distilled water were added. The growth regulators used were  $\alpha$ -naphthaleneacetic acid (NAA), 6-(furfurylamino)-purine (kinetin), gibberellic acid, *p*-chlorophenoxyisobutyric acid (PCPIB), coconut milk, and  $\alpha$ -(1-naphthylmethylsulphide)-propionic acid (NMSP).

The tryptophan and coconut milk solutions were sterilized by filtration, the others by autoclaving.

Observations on nodulation were made daily and the number of days from sowing to the formation of the first macroscopically visible nodule (nodule initiation) was recorded. At 42 days from sowing the plants were harvested and the total number of nodules on each plant was recorded, and in some cases nodule volume and root dry weight were also measured.

#### (b) *Inoculation of Test Legumes with Rhizobium*

Plants were inoculated from 7-day-old cultures at the rate of approximately  $10^6$  cells per tube. Strains used were *Rh. trifolii* strain A, *Rh. trifolii* Bart A (a non-infective mutant of strain A), *Rh. meliloti* AH<sub>2</sub> (all from Rothamsted Experiment Station, England), *Rh. meliloti* SU277, *Rh. trifolii* SU297, and *Rh. japonicum* CC705.

Immediately prior to inoculation, streak plates on yeast mannitol agar were made from the root medium and tubes thus shown to be contaminated were excluded from the assays.

#### (c) *Shake Cultures of Nodule Bacteria*

Flasks containing 250 ml of the medium of Norris (1958) with or without  $4 \times 10^{-6}$  M L-tryptophan were inoculated, where necessary, with *Rh. trifolii* SU297 and were shaken at 25°C in darkness for 7 days.

(d) *Detection of Tryptophan*

Subterranean clover root medium from inoculated (SU297) and non-inoculated cultures was collected, extracted three times with ether, and the aqueous layer was evaporated to dryness under reduced pressure. The residue was extracted with hot ethanol and the alcohol solution was filtered and evaporated to dryness under reduced pressure. This residue was dissolved in 5 ml of a solution of mineral salts and glucose in distilled water and was sterilized by filtration through sintered glass. Distilled water controls were similarly treated and standards containing  $10^{-6}$ ,  $3 \times 10^{-6}$ ,  $10^{-5}$  M L-tryptophan were prepared. In early experiments, solutions were inoculated with the tryptophan-requiring mutant *Salmonella typhimurium* strain T<sub>1</sub>, and in later experiments with the tryptophan- or indole-requiring mutant *Pseudomonas aeruginosa* strain 1-4 (kindly provided by Dr. B. W. Holloway, University of Melbourne). Any indole present in the root medium was removed by the preliminary ether extraction. Cultures were held at 37°C for 24 hr, then bacterial growth was measured as optical density at 700 m $\mu$  using a Unicam spectrophotometer.

(e) *Assay for Auxin*

Bulked growth liquid from 30 or more water-culture tubes or shake-culture liquid was brought to pH 3.5 with phosphoric acid, then extracted three times with peroxide-free ether. The ether was evaporated off and the residue dissolved in 2 ml 1.5 per cent. agar. From this, blocks 10 mm<sup>3</sup> were obtained and used in the *Avena* curvature method of auxin assay, carried out exactly as described by Zwar and Rijven (1956).

(f) *Chromatography of Auxins*

The residues from the evaporation of ether extracts obtained in the previous section were chromatographed on No. 1 Whatman filter paper. Guide chromatograms of synthetic IAA were also run and the  $R_F$  ranges of these guide spots in the solvents used were as follows: isopropanol-water-30 per cent. ammonia (10 : 1 : 1 v/v),  $R_F$  0.3-0.45; methanol-water-30 per cent. ammonia (10 : 1 : 1 v/v),  $R_F$  0.5-0.65; and water,  $R_F$  0.85-0.9.

Chromatograms were treated in one of the following ways:

- (1) Sprayed with Salkowski or Ehrlich reagents (Stowe and Thimann 1954);
- (2) Cut into portions and the auxins in each portion assayed by the *Avena* coleoptile section test (Kefford 1955);
- (3) The areas of the chromatograms corresponding with IAA markers were eluted with ethanol, the alcohol evaporated, and the residue dissolved in agar solution for assay in the *Avena* curvature test.

### III. RESULTS

(a) *Exudation of Tryptophan by Subterranean Clover Roots*

The results in Table 1 show that tryptophan was detected in the concentrated growth medium of both inoculated and uninoculated subterranean clover. These results obtained with *Pseudomonas aeruginosa* strain 1-4 were supported by similar

results obtained with *Salmonella typhimurium* strain T<sub>1</sub>. The lower tryptophan content in the inoculated medium was presumably partly due to conversion to IAA by the nodule bacteria.

TABLE 1

OCCURRENCE OF TRYPTOPHAN IN ROOT EXUDATES OF INOCULATED (STRAIN SU297) OR UNINOCULATED SUBTERRANEAN CLOVER, AS MEASURED BY THE GROWTH OF A TRYPTOPHAN-REQUIRING STRAIN OF *PSEUDOMONAS AERUGINOSA*

Growth is expressed as optical density (at 700 m $\mu$ ) of the culture and the results of duplicate experiments (I and II) are given

Addition to <i>Pseudomonas</i> Medium	Growth of <i>Pseudomonas</i> (optical density $\times 10^3$ )	Addition to <i>Pseudomonas</i> Medium	Growth of <i>Pseudomonas</i> (optical density $\times 10^3$ )
Tryptophan $10^{-6}$ M	6	Root exudate	
Tryptophan $3 \times 10^{-6}$ M	32	SU297 present (I)	5
Tryptophan $10^{-5}$ M	48	SU297 present (II)	25
Clover growth medium (I)	0	SU297 absent (I)	43
Clover growth medium (II)	0	SU297 absent (II)	46

(b) *Production of Auxin by Subterranean Clover and Associated Nodule Bacteria*

Data from tests 1 and 2 in Table 2 show that, when subterranean clover plants were grown alone on distilled water, no auxin was detected in extracts of the root

TABLE 2

AVENA COLEOPTILE CURVATURES GIVEN BY EXTRACTS OF ROOT GROWTH MEDIUM FOLLOWING CULTURE OF SUBTERRANEAN CLOVER PLANTS INOCULATED WITH THE RHIZOBIUM STRAINS INDICATED

The curvatures given by a pure agar control and by IAA at a concentration of  $7.5 \times 10^{-8}$  g/ml are also shown

Test No.	Inoculant	No. of Tubes Pooled	Curvature by Extract (degrees)	Curvature by Control (degrees)	Curvature by IAA (degrees)
1	Uninoculated	44	-0.7	+0.3	-22.1
1	Clover strain SU297	44	-28.1	+0.3	-22.1
2	Uninoculated	48	0.0	+1.0	-27.7
3	Clover strain SU297	25	-14.2	0.0	-26.6
4	Clover strain A	82	-25.6	+0.4	-26.1
4	Clover strain Bart A	79	-21.3	+0.4	-26.1
5	Clover strain SU297	44	-20.9	-1.3	-29.0
5	Lucerne strain AH <sub>2</sub>	41	-13.9	-1.3	-29.0
5	Soybean strain CC705	28	-2.0	-1.3	-29.0
6	Soybean strain CC705	46	0.0	-0.7	-29.7

growth medium. If, however, for the last 3 days strain SU297 was present in the culture, auxin could be detected (tests 1, 3, and 5, Table 2). This auxin produced



curvatures at a similar distance down the *Avena* coleoptiles to that given by IAA, showing that it was readily transported. The auxin activity had the same  $R_F$  values as IAA in three solvent systems (Table 3) and auxin activity could not be detected on the chromatograms other than in the IAA position.

TABLE 3

AVENA COLEOPTILE CURVATURES GIVEN BY ELUATES OF THE AREAS CORRESPONDING WITH IAA GUIDES AND THE REMAINDER OF CHROMATOGRAMS OF EXTRACTS OF THE ROOT GROWTH LIQUID OF SUBTERRANEAN CLOVER PLANTS INOCULATED WITH STRAIN SU297

The solvents used to develop the chromatograms and curvatures given by a pure agar control and IAA at a concentration of  $7.5 \times 10^{-8}$  g/ml are also shown

Chromatographic Solvent	Curvature by Eluate of IAA Guides (degrees)	Curvature by Eluate of the Remainder (degrees)	Curvature by Control (degrees)	Curvature by IAA (degrees)
<i>iso</i> Propanol-ammonia	-23.2	-0.3	+1.0	-27.7
Methanol-ammonia	-22.2	—	0.0	-26.6
Water	-13.1	-1.8	0.0	-26.6

Extracts of shake cultures of SU297 grown in the presence of tryptophan produced an auxin with the same  $R_F$  value as IAA in the solvents *isopropanol*-ammonia and water. When sprayed with the Salkowski or Ehrlich reagents, similar chroma-

TABLE 4

AVENA COLEOPTILE CURVATURES GIVEN BY EXTRACTS OF ROOT GROWTH LIQUIDS WHICH CONTAINED THE CONCENTRATIONS OF POTASSIUM NITRATE SHOWN, AND IN WHICH SUBTERRANEAN CLOVER PLANTS, INOCULATED WITH STRAIN SU297, HAD GROWN

Conventions as in Table 2. The effects of the same concentrations of potassium nitrate upon time to nodulation are also shown

Test No.	Potassium Nitrate Concn. (%)	Days to First Nodule	Number of Tubes	Curvature by Extract (degrees)	Curvature by Control (degrees)	Curvature by IAA (degrees)
5	0.0	4.3	45	-20.9	-1.3	-29.0
5	0.05	8.4	44	-22.4	-1.3	-29.0
6	0.05	8.4	39	-24.8	-0.7	-29.7
6	0.1	8.6	41	-23.5	-0.7	-29.7
6	0.2	9.9	32	-26.6	-0.7	-29.7

tograms held spots of the same colour and  $R_F$  value as IAA, and colour was detected only at the IAA position. Using the *Avena* section assay, no IAA could be detected in extracts of shake cultures of SU297 not containing tryptophan.

The non-infective strain, Bart A, produced auxin in the presence of subterranean clover roots to the same extent as the infective parent, strain A (Table 2, test 4). The lucerne strain AH<sub>2</sub> produced auxin in the presence of clover roots (Table 2, test 5), but the soybean strain CC705 did not (Table 2, test 5), even when

TABLE 5

INFLUENCES OF SOME PLANT GROWTH REGULATORS UPON THE NODULATION BEHAVIOUR OF LUCERNE PLANTS INOCULATED WITH STRAIN AH<sub>2</sub>

For the treatments in which some plants failed to nodulate, the mean times to nodule initiation and the mean numbers of nodules per plant were calculated on nodulated plants only

Treatment	Concn. (M)	Plants Nodulated (%)	Nodule Initiation (days)	Nodule Number per Plant Nodulated	Nodule Volume (mm <sup>3</sup> )		Root Weight (mg)
					Mean per Plant	Mean per Nodule	
Experiment 1							
Control		100	10.8	8.5	9.7	1.14	7.0
NAA	10 <sup>-6</sup>	30	32.0***	3.7***			
NAA	10 <sup>-7</sup>	53	14.8**	5.6**	10.6	1.89	6.2
PCPIB	10 <sup>-5</sup>	100	10.7	12.6***	12.3*	0.98	7.1
PCPIB	10 <sup>-6</sup>	100	11.7	9.8			
Gibberellic acid	10 <sup>-6</sup>	100	13.3**	5.1***	9.6	1.88	6.3
Gibberellic acid	10 <sup>-8</sup>	100	11.4	9.2			
Kinetin	10 <sup>-8</sup>	58	12.8*	10.0			
Kinetin	10 <sup>-9</sup>	100	13.0**	10.0			
Coconut milk	5%	100	13.8***	8.8			
Coconut milk	1%	100	10.8	10.9			
Experiment 2							
Control		100	13.7	12.7			
NAA	10 <sup>-8</sup>	100	12.9	12.7			
Kinetin	10 <sup>-6</sup>	40	32.0***	5.0***			
NMSP	10 <sup>-5</sup>	100	13.2	11.8			
NMSP	10 <sup>-6</sup>	100	14.0	12.7			
NMSP	10 <sup>-7</sup>	100	13.4	13.8			

\* Difference from control significant at  $P = 0.05$ .

\*\* Difference from control significant at  $P = 0.01$ .

\*\*\* Difference from control significant at  $P = 0.001$ .

plants and bacteria were cultured together for 6 days instead of the usual 3 days (Table 2, test 6). Table 4 shows that the presence of potassium nitrate in the root growth medium delayed nodulation, but did not affect the production of auxin by subterranean clover seedlings and strain SU297.

### (c) Influences of Growth Regulators and Tryptophan on the Nodulation of Lucerne

In Table 5 are shown the results of two experiments in which growth regulators were added to cultures of lucerne plants inoculated with strain AH<sub>2</sub>.



The auxin NAA, in concentrations down to  $10^{-7}\text{M}$ , caused a delay in the initiation of nodules and a decrease in the number of nodules formed and prevented many plants from forming any nodules. At high concentrations, NAA caused an obvious inhibition of root growth, but at  $10^{-7}\text{M}$  the dry weight of roots was the same as controls and the nodule number was still reduced. The volumes of individual nodules on plants treated with  $10^{-7}\text{M}$  NAA were larger than the controls and this resulted in the nodule volume per plant being the same for NAA-treated plants and control plants. NAA was used in these tests because IAA is decomposed by light and plant enzymes.

Treatment with the antiauxin PCPIB did not influence nodule initiation, but, at  $10^{-5}\text{M}$ , nodule number and nodule volume per plant were increased. The substance NMSP which has been reported to promote root growth (Street 1954), did not influence nodule initiation or number.

Treatment with  $10^{-8}\text{M}$  kinetin prevented half of the plants from nodulating, but those that nodulated produced the first nodule at the same time as the controls. Nodule number per nodulated plant was unaffected by kinetin at  $10^{-8}$  or  $10^{-9}\text{M}$ . Concentrations of  $10^{-6}\text{M}$  or higher caused visible inhibition of the root main axis growth and lateral development. Few such plants nodulated and on those that nodulated, the nodules were fewer and appeared later than on control plants.

Gibberellic acid at  $10^{-6}\text{M}$  slightly delayed nodule initiation and greatly reduced nodule number. Root weight and nodule volume per plant, however, were unchanged by this treatment.

Coconut milk at 5 per cent. concentration caused a delay of nodule initiation but did not affect nodule number; 1 per cent. coconut milk produced no effects.

In Table 6 are presented a selection of results of experiments, in which the effects upon nodule initiation of the addition of L-tryptophan to cultures of lucerne inoculated with strain AH<sub>2</sub> and barrel medic inoculated with strain SU277 were observed. At concentrations above  $10^{-5}\text{M}$ , tryptophan treatment clearly delayed nodulation. At concentrations of  $10^{-7}\text{M}$  or  $2 \times 10^{-8}\text{M}$  a slight hastening of nodulation was observed in tests B, C, E, H<sub>2</sub>, and K<sub>2</sub> but overall a statistically significant hastening was not established.

#### IV. DISCUSSION

##### (a) *Symbiotic Synthesis of Auxin*

It has been shown that an auxin is produced when subterranean clover seedlings and nodule bacteria are cultured together, but in the absence of bacteria no auxin is produced. Since nodule bacteria do not produce auxin in the absence of tryptophan (see also Georgi and Beguin 1939) and clover and other legume roots exude tryptophan (Rovira 1956; Dehay and Care 1958), it can be concluded that in the absence of clover roots no auxin would be produced. Hence the presence of both nodule bacteria and clover roots was essential for the production of auxin in the root medium.



(b) *Identity of the Auxin*

Although IAA has not been isolated from cultures of nodule bacteria, there is considerable evidence that it is the auxin produced. The biological assay used here, the *Avena* curvature test, involves the polar transport of a substance down the coleoptile for a "deep" curvature to be obtained. The auxin transport system has been shown to be highly specific, in general only IAA or substances readily converted to it being transported (Zwar and Rijven 1956). There is additional chromatographic evidence from extracts of root medium and shake cultures of nodule bacteria. In each of these, the auxin activity had the same  $R_F$  value as IAA and activity was detected only at this  $R_F$ . On chromatograms of extracts of shake cultures, a substance giving the correct colour reactions was detected at the IAA position.

Assuming the auxin is IAA, its concentration in the root medium of 21-day-old subterranean clover seedlings inoculated for 3 days with SU297 can be roughly estimated as  $5 \times 10^{-7}$  g/l. This value, however, has doubtful significance owing to the destruction of IAA in the root medium by light and perhaps by other means. Tryptophan would be similarly destroyed, thus the rough estimate of its concentration as  $7.5 \times 10^{-6}$  g/l in root medium of uninoculated clover seedlings, also has little quantitative significance. The destruction of indole derivatives in the root medium would be accelerated by the presence of substances such as riboflavin and of heavy metals. For the latter reason it is an advantage to be able to grow seedlings in water.

Since it is probable that, as in higher plants (Gordon 1956) and in other micro-organisms (Brian 1957), *Rhizobium* produces IAA from tryptophan, it can be concluded that when clover roots and nodule bacteria are cultured together, tryptophan, exuded by the roots, is converted to IAA by the nodule bacteria.

Tests for growth activity and colour reactions on chromatograms of extracts of *Rhizobium* SU297 shake cultures revealed only one growth-active area and one area giving colour reactions of indole derivatives. Pate (1958) found chromatographically that clover nodules contained three auxins. One of these was probably IAA; the others, according to their  $R_F$  values, may have been conjugation compounds of IAA with amino acids or ammonia. Such conjugation compounds are formed in nodules treated with IAA *in vitro* (Andreae, personal communication) and the reactions are considered to be for the detoxification of IAA. If these reactions occur normally in nodules it would be the first demonstration of a natural function for them. Their existence in nodules could also explain the ability of these root tissues to tolerate the abnormally high concentrations of auxin found in nodules, in some respects nodules being physiologically equivalent to lateral roots (Nutman 1956). Of course, other reactions such as oxidation of IAA or the auxin transport system may have roles in localizing the effects of nodule auxin.

(c) *Possible Roles for Auxin in Infection Thread and Nodule Growth*

Nutman (1956) and Brian (1957) consider that the role of auxin in nodulation is the curling of root hairs. We suggest that auxin may also play an important

role in the growth of the infection thread and the initiation of the cell division leading to nodule formation. A consideration of these growth processes in the light of the known growth effects of IAA provides support for this suggestion. The sequence of events could be envisaged as follows:

Morphological studies show that the infection thread is a sealed bag inside the root hair and that its wall is of host plant origin (Nutman 1956). Since this bag is extending, pressure inside it must be greater than the pressure inside the root hair. It is postulated that a colony of nodule bacteria on a root hair could do two things: first, it could produce IAA which renders the cell wall plastic; secondly, it could produce osmotic conditions which would cause the root-hair cytoplasm to retreat from the colony. Repetition of these processes, as the bacteria follows the growth of the thread, could account for growth of an infection thread through a root-hair cell. Growth into an adjacent cell could result from the retreat of a neighbouring cytoplasm at a pit area where the cytoplasms are continuous and the cell wall is still capable of being loosened by IAA.

The cell divisions in the root cortex, which give rise to the nodule, could also be initiated by IAA produced by nodule bacteria. For cell division to be initiated in many tissues, a kinin and an auxin are required (Skoog and Miller 1957). Thus cell division could be initiated when the infection thread or actually the IAA produced in it approached a cell containing the required concentration of a kinin. The initial division in nodule formation is by a cell differing from its neighbours in being disomatic.

The above conjecture relates only to the *local* production of IAA at the point of entry of a bacterial colony into a root hair and subsequently in the infection thread. Our results indicate that the production of IAA *generally* in a liquid growth medium of restricted volume does not promote the effects postulated. In this situation IAA appears to inhibit nodule development by virtue of its general inhibitory action on root development. It was hoped that with the addition of graded amounts of tryptophan, a condition might be reached in which localized IAA production at the root-hair surface might be stimulated, before the overall concentration of IAA in the medium reached an inhibitory level. However, only slight and statistically insignificant stimulation was found at any level in the tryptophan concentration series. It is probable that under the culture conditions used, a localized build up in IAA concentration was not possible, or that tryptophan concentration was not limiting infection.

#### (d) *Production of IAA under Conditions Unfavourable to Nodulation*

In two situations where clover plants and nodule bacteria were cultured together, but where nodules would not be formed, IAA was still detected (Table 2). These situations were: (1) clover roots with Bart A, the non-infective mutant of a clover strain, (2) clover roots with lucerne strain AH<sub>2</sub>. In a third situation (clover roots with strain SU297 in the presence of nitrate, where nodulation was substantially delayed) IAA was again readily detectable (Table 4). In these cases IAA production cannot be the block in the complex of processes that contribute to nodule formation.

When clover seedlings were cultured with soybean strain CC705, no auxin was detected in the root medium. Also these bacteria were found not to convert tryptophan to IAA in shake culture. Both negative results may result from the slow relative growth of this class of bacteria (Burris and Wilson 1939). The bacteria would appear capable of synthesizing IAA because we have found the nodules of soybean, cowpea, and peanut to contain IAA at approximately 50 mg/kg fresh nodule tissue.

#### (e) *Effects of Growth Regulators upon Nodulation*

The survey of the effects of the addition of a variety of growth regulators to legume growth media (Table 5) produced no clear leads to roles for these substances in nodulation. But some of the results obtained may be relevant to experiments done under the same conditions of restricted volume of medium, namely preplanting experiments. Nutman (1953, 1957) observed the nodular behaviour of plants grown on media in which other legumes had previously grown. Short periods of preplanting hastened the formation of the first nodule, while longer periods caused a diminution in the rate of formation of subsequent nodules until even fewer nodules were formed than on non-preplanted controls. Our results have shown that preplanting would add tryptophan to the medium and nodule bacteria would convert this to IAA. Also the auxin NAA added to the medium reduces the number of nodules formed, while addition of the antiauxin PCPIB increases this number. We suggest that the number of nodules produced in media of restricted volume is limited by the auxin accumulated under these conditions and that the inhibitory preplanting effects are due to auxin. The observed hastening of nodulation by preplanting has been shown to be at least in part due to the removal of traces of nitrate from the medium (Gibson 1959).

### V. ACKNOWLEDGMENTS

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# THE ELECTRIC RESISTANCE OF THE CELL MEMBRANES IN A *CHARA* AND A *NITELLA* SPECIES

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## Summary

Experiments with microelectrodes are described which demonstrate that the plasmalemma (outer cytoplasmic membrane) is responsible for most of the direct current resistance in cells of *Nitella*. Further experiments show that the microelectrode method gives reliable measurements of the membrane resistance in such cells. The resistance of the plasmalemma, in the young *Nitella* cells used, and in various media, ranged from 5 to 50  $\text{k}\Omega \text{ cm}^2$ , while the resistance of the tonoplast was less than 3  $\text{k}\Omega \text{ cm}^2$ . The combined membrane resistance of the mature *Chara* cells used ranged from 10 to 30  $\text{k}\Omega \text{ cm}^2$  with a mean value of 15  $\text{k}\Omega \text{ cm}^2$ , the cells being bathed in 1  $\text{mM}$  NaCl solution.

It is shown that the results cannot be interpreted in terms of a model proposed by Briggs (1957) which did not include a plasmalemma. The relationship between this work and recent studies of ionic fluxes in the Characeae is discussed.

## I. INTRODUCTION

Many investigators have studied the large cylindrical internodal coenocytes ("cells") of the Characeae by physiological and biophysical methods. Among them, a number have measured the resistance to an electric current which flows across the surface of the cell from vacuole to bathing medium. Such measurements have been reported by Blinks (1930, 1936), Umrath (1940), Weidmann (1949), Bennett and Rideal (1954), and Findlay (1959). In all these cases, with the possible exception of Umrath's work, the resistance measured was that of the peripheral layer of cytoplasm, together with its bounding membranes, the plasmalemma and the tonoplast. (This is called here the "combined membrane resistance".)

As a result, they have offered no test of the suggestion (Hope and Robertson 1953; Briggs and Robertson 1957) that the plasmalemma does not constitute a diffusion barrier in plant cells.

The ability to insert microelectrodes into the cytoplasm and the sap of these cells (Walker 1955) offers the possibility of testing this suggestion. As stated in a preliminary report (Walker 1957) it has been possible to measure the resistance of the plasmalemma, and to show that it accounts for most of the resistance of the cell surface. The possibility that the use of microelectrodes for resistance measurements gives grossly low values has been ruled out by further experiments which are reported. These measurements and their significance are more fully discussed here, in the light of criticisms by Briggs (1957, 1958), and of recent measurements of ionic fluxes (MacRobbie and Dainty 1958; Diamond and Solomon 1959).

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## II. EXPERIMENTAL

The plants used were *Chara australis* R. Br. var. *australis*, and an unknown species of *Nitella*. (The tentative identification of this species as *N. gloeostachys* has been withdrawn.) The plants were grown in tap water-pond mud cultures in the laboratory, in indirect daylight. Internodal cells for experiment were isolated from their neighbouring cells and kept for several days in dilute salt solutions before use.

The microelectrode technique was similar to that already described (Walker 1955), except that two microelectrodes were inserted into the cell.

The first experiments were designed to separate the resistances of the tonoplast and plasmalemma. The pretreatment and the experimental bathing medium varied from cell to cell and from time to time with one cell, as the absolute values of membrane resistance were not considered important. The solution used during the measurement was 0.1 mN or 1 mN KCl, 1 mN NaCl, or 1 mN  $\text{CaCl}_2$ .

It was important to locate the tip of the microelectrode by observation, so young transparent cells of *Nitella* were used.

The electric resistance associated with the surface of the cell was measured by passing a known direct current across it, the resulting change in potential difference across the surface being measured. The current flowed from the tip of a microelectrode in the vacuole, across the surface of the cell, and to a reference electrode in the bathing medium. A second inserted microelectrode, and the same reference electrode, served for measurements of potential difference (p.d.).

For each measurement, after a reading of the normal membrane p.d., the current was switched on for a few seconds, and the first steady potential reading was taken. For each cell measurements were made:

- (1) With the tip of the potential-measuring electrode in the outside medium near the cell wall: this gave the "external resistance" which was subtracted from the later readings;
- (2) With the tip in the flowing cytoplasm: this gave the resistance due to the plasmalemma; and
- (3) With the tip in the vacuole: this gave the resistance due to the tonoplast and plasmalemma in series.

The current used was so small that the plant membranes behaved essentially as ohmic systems, with a resistance independent of the measuring current. Usually the change in membrane p.d. produced by the current was of the order of 10 mV, and the current was of the order of 10  $\mu\text{A}$ . Experiments confirmed that at this level the membrane resistance did not change significantly with the magnitude or direction of the current (Hope and Walker, unpublished data).

The inserted microelectrode tips were usually about 0.1 mm apart, and close to the middle of the cell's length. They were micro salt-bridges of the usual type, filled with 0.3N KCl, and about 2–5  $\mu$  in diameter at the tip.

The reference electrode, initially an agar salt-bridge, was later a long coil of chloride-coated silver wire, parallel to and close to the cell.

A Kin-Tel electronic galvanometer (model 204A) was used to measure the current, which was derived from an adjustable potential source in series with a high value resistor (typically 20 V and 10 kM $\Omega$ ). Potential difference measurements were made initially with a valve electrometer of high input resistance, and later with a vibrating condenser electrometer (E.I.L. Vibron model 33B). A chart recorder was used with the latter instrument. The current measurements were accurate to about  $\pm 3$  per cent., and the potential measurements to about  $\pm 0.5$  mV. The accuracy of the complete resistance measurement is estimated to be of the order  $\pm 10$  per cent.

To make sure that the microelectrode insertion was not radically altering the resistance of one or other of the membranes, a comparison series of experiments was done. Larger cells were necessary, and internodes of *C. australis*, 1–4 cm long, were

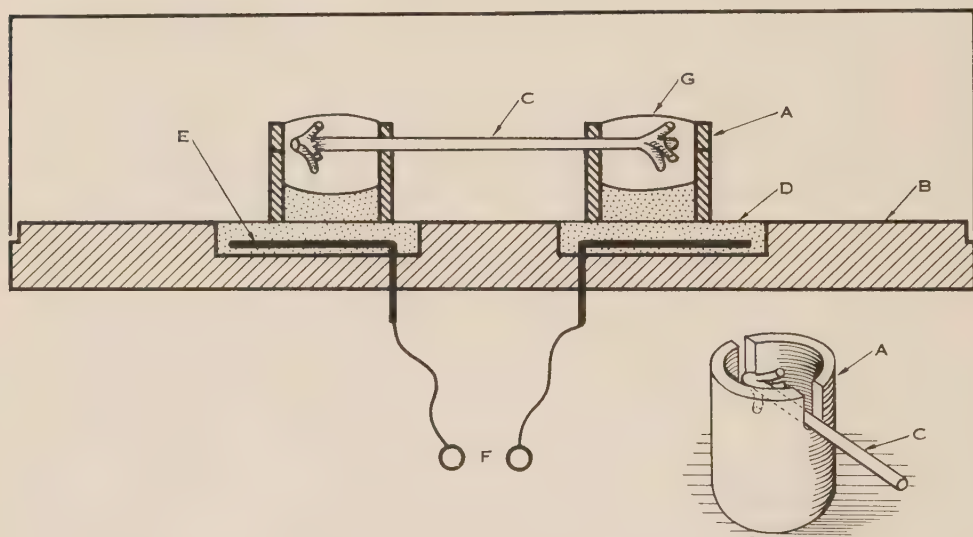


Fig. 1.—Apparatus (not to scale) for resistance measurements using external contacts. *A*, open polythene cylinders, about 1 cm diameter, with diametrically opposed grooves; *B*, polythene base; *C*, internodal cell of *Chara*; *D*, block of agar inset into base; *E*, silver-silver chloride electrode; *F*, leads to electrometer; *G*, pools of solution bathing the cell.

used. An arrangement of external liquid contacts (like that of Blinks 1930) was used to measure the resistances of the two membranes in series. The same measurement was then made on each cell by means of microelectrodes inserted into the vacuole. The two measurements were made within 1 or 2 hr, using the same bathing medium of 1.0 mN NaCl, in which the cells had been kept for at least 24 hr. This bathing medium was chosen as giving stable resistance values.

The liquid-contact apparatus is shown in Figure 1. Two external contacts were used, current entering the cell at one and leaving at the other. Chloride-coated silver wires were embedded in agar, which was in contact with the solution pools. These pools were stagnant, but the solution was exchanged at frequent intervals.

The direct current (d.c.) resistance of the apparatus with a cell in place was measured, as before, by measuring the change in p.d. between the terminals when a known small current was passed. The resistance at 10 kc/s was then measured with the alternating current (a.c.) bridge of de Plater and Greenham (1959). This resistance was equal to the d.c. resistance less the membrane resistances (which at 10 kc/s are shunted by the low capacitive reactance of the membrane\*). This equality was checked by d.c. measurements on a chloroform-killed cell. The difference between the d.c. and 10 kc/s readings was therefore taken as the resistance of the membranes. This of course was the sum of the resistances of the areas covered by the liquid contacts. It was found that the shunt resistance of the cell wall was sufficiently high to be neglected, with a consequent error of less than 10 per cent.

TABLE 1  
MEMBRANE RESISTANCES IN YOUNG CELLS OF NITELLA  
An asterisk indicates the measurement made first

(a) Measurements Separated by 1 Hr			(b) Measurements in Quick Succession		
Cell Serial No.	Resistance of Plasmalemma (k $\Omega$ )	Sum of Resistances of Plasmalemma and Tonoplast (k $\Omega$ )	Cell Serial No.	Resistance of Plasmalemma (k $\Omega$ )	Sum of Resistances of Plasmalemma and Tonoplast (k $\Omega$ )
R13	210*	200	R13	400	400*
R13	370	470*	R20	270	260*
R16a	730	880*	R21	290	330*
R18	300	400*			
R21	300*	370			
R21	340	350*			

Measurements on each cell were made at a number of different values of contact length and gap length. Diameter and length were measured with an eyepiece graticule in a Zeiss stereomicroscope. From each pair of d.c. and a.c. resistances, the combined membrane resistance  $r$  (in k $\Omega$  cm<sup>2</sup>) could be calculated. This is fully discussed by Blinks (1930). The average combined membrane resistance for each cell was found, averaging over the values found for different gap and contact lengths.

During the early experiments the room temperature ranged from 17 to 25°C; during the comparison experiments the room temperature was constant at 20°C.

### III. RESULTS

In the first type of experiment, the microelectrode for current conduction was inserted into the cell vacuole, its tip being near the axis of the cell. The potential-measuring electrode was inserted into the cytoplasm or the vacuole, and as soon as possible after the resistance measurement it was withdrawn and inserted into the

\* Curtis and Cole (1937). Further confirmation was the finding that the impedances measured at 1 kc/s and at 10 kc/s were equal and essentially resistive.



other phase. Thus for each cell, measurements were made in succession of the plasmalemma resistance, and of the sum of the plasmalemma and tonoplast resistances. The interval between the measurements was of the order of 1 hr, as the electrode was not withdrawn until the seal had formed. This minimized injury by preventing loss of cell contents. The results of these experiments are given in Table 1(a). In a few favourable cases the tip of the potential electrode was in the vacuolar sap, and it was then covered by the flowing cytoplasm. This enabled the two measurements to be made in quick succession. The results of these experiments are given in Table 1(b).

It is clear that in these cells the greater part of the d.c. resistance is due to the plasmalemma. The tonoplast resistance appears to vary from 0 to 100 k $\Omega$ , but

TABLE 2  
PLASMALEMMA RESISTANCES OF CELLS OF NITELLA

Cell Serial No.	Plasmalemma Resistance (k $\Omega$ cm <sup>2</sup> )	Bathing Medium (1.0 mN in each case)	Cell Serial No.	Plasmalemma Resistance (k $\Omega$ cm <sup>2</sup> )	Bathing Medium (1.0 mN in each case)
R16a	6.1 13 21	KCl NaCl CaCl <sub>2</sub>	R20	7.8 8.7	NaCl Culture medium
R17	2.0	KCl	R21	5.4 12	NaCl CaCl <sub>2</sub>
R18	6.9	KCl	R22	30 63	KCl CaCl <sub>2</sub>

it is not clear how much of this is experimental error. The most reliable results (Table 1(b)) show low resistances for the tonoplast.

To compare measurements on different cells, it is necessary to express results in terms of the surface resistance  $r$  (in k $\Omega$  cm<sup>2</sup>) and the product of the measured resistance  $R$  and the surface area  $A$ :

$$r = R \cdot A \dots \dots \dots (1)$$

This involves the assumptions that the cell surface is of uniform resistance, and that cells of different sizes differ in their surface areas and not in the specific properties of their membranes. In spite of these assumptions this procedure must be adopted for the present. Equation (1) is true only if the current density is uniform over the cell surface. In the present experiments the longitudinal resistance of the cell sap causes the current density to fall off towards the ends of the cell. It can be shown (see Appendix I) that an approximate expression for  $r$  in terms of measurable quantities is

$$r \cong [R - (R_L/12)]A, \dots \dots \dots (2)$$

where, as before,  $R$  is the measured resistance, and where  $R_L$  is the longitudinal resistance of the cylinder of vacuolar sap. The approximation is good for  $R_L < 10r/A$ ,

which condition is easily met in the *Nitella* cells studied. In fact,  $R_L/12$  is a small correction, and a plausible estimate of  $R_L$  was sufficient. Taking the sap to have a resistivity of  $50 \Omega \text{ cm}$ , the membrane resistance ( $r$ ) of the plasmalemma was calculated for a number of cells (see Table 2). For comparison, the membrane resistance of the tonoplast for the cells of Table 1 appears to vary from 0 to  $3 \text{ k}\Omega \text{ cm}^2$ , with an average value of about  $1 \text{ k}\Omega \text{ cm}^2$ . Since pretreatments were not standardized, and the cells varied considerably in size, it is not surprising that there is considerable

TABLE 3  
MEMBRANE RESISTANCES OF CELLS OF CHARA BY TWO METHODS

Cell Serial No.	Combined Membrane Resistance ( $\text{k}\Omega \text{ cm}^2$ )		Ratio
	By External Contact Method	By Microelectrode Method	
C8	28	21	1.33
C9	11	14	0.79
C10	6	8.5	0.71
C11	6	10	0.60
C13	24	17	1.41
C14	31	32	0.97
C15	19	20	0.95
C16	12	10	1.20
C18	11.5	10.5	1.10
C19	10	9.5	1.05
C20	8.5	10.5	0.81
Mean	15.2	14.8	

variation in the results in Table 2. However, there is a consistently lower relative resistance in KCl solutions than in NaCl or  $\text{CaCl}_2$  solutions. A similar effect, but of much greater magnitude, was reported by Blinks (1930).

The results of the comparison between the microelectrode method and the external contact method are given in Table 3. The values of  $r$  in this table have again been calculated from equation (2), using values of  $R_L$  determined directly from the a.c. resistance measurements. This was necessary as the correction term was a significant part of the measured resistance. Table 3 shows that there is some variation between the results of the two methods, which can perhaps be attributed to the unavoidable handling of the cells. However, the agreement between the two methods is fair, and the agreement between the mean values they yield is good. It is clear that the microelectrode method does not involve large artefacts or errors due to injury of the cell.

## IV. DISCUSSION

These experiments show the greater part of the d.c. resistance of *Nitella* cells to be located between the flowing cytoplasm and the bathing medium. The most reasonable conclusion is that this part of the resistance is due to the plasmalemma,\* or outer cytoplasmic membrane. The longitudinal electric resistance of the cell wall is consistent with a transverse resistance of at most a few ohm cm, so that the cell wall cannot be responsible for the resistance found.

The cytoplasm was suggested by Briggs (1957, 1958) as possibly the seat of the resistance. He proposed a model of the cell in which the cytoplasm was a negatively charged Donnan system, and the tonoplast was impermeable to cations. This model was stated to be consistent with the finding that the greater part of the cell's d.c. resistance was between the cytoplasm and the bathing medium. However, there is

TABLE 4  
MEMBRANE RESISTANCES IN CHARACEAE

Author	Method	Species	Mean Membrane Resistance ( $k\Omega\text{ cm}^2$ )
Blinks (1930)	External contacts	<i>Nitella flexilis</i>	100–250
Blinks (1930)	External contacts	<i>Chara coronata</i>	50–100
Umrath (1940)	Microelectrodes	<i>Nitella mucronata</i>	100*
Bennett and Rideal (1954)	Microelectrode (a.c.)	<i>Nitella</i> sp.	80
Findlay (1959)	Microelectrodes	<i>Nitella</i> sp.	35
Walker (present work)	Microelectrodes	<i>Nitella</i> sp.	5–50
Walker (present work)	Microelectrodes	<i>Chara australis</i>	15
Walker (present work)	External contacts	<i>Chara australis</i>	15

\* One determination only.

good reason to think that the cytoplasm has a high electric conductivity (Walker 1957), and Briggs has not shown, but merely stated, that the presence of a cation-impermeable tonoplast could modify this. A simple treatment of his model (Appendix II) shows that his statement is not correct and that his model is inconsistent with the results reported here.

Since the earlier publications, studies of ionic fluxes in the Characeae by MacRobbie and Dainty (1958) and Diamond and Solomon (1959) have provided further evidence for the existence of a plasmalemma with considerable diffusion resistance. This work has shown that the flux of ions across the plasmalemma is not much greater than the electric resistance suggests. There is therefore no anomaly which would require the ion-carrier hypothesis to be applied to the plasmalemma (Walker 1957), at least as far as sodium and potassium ions are concerned. There is, however, a serious anomaly (MacRobbie and Dainty 1958) between the measured

\* In spite of recent objections to this usage (Höfler 1959) it seems just as reasonable to apply "plasmalemma" to the outer cytoplasmic membrane as it is to apply "tonoplast" to the vacuolar membrane.

tonoplast resistance (about  $1 \text{ k}\Omega \text{ cm}^2$ ) and the value calculated from ion fluxes (e.g.  $250 \text{ k}\Omega \text{ cm}^2$ ).

It is possible to tabulate values of  $r$  obtained by a number of different workers, as shown in Table 4. Detailed comparison of these results is pointless, as they refer to different species and different pretreatments and bathing solutions. However, it may be noted that the high values of Blinks were obtained only after long soaking in distilled water. He strove to get high values, which he seems to have regarded as "normal" and indicative of good health. There is little evidence that such high values are in any way normal.

We may conclude that there is no special reason to assume that the high values are typical—as MacRobbie and Dainty have assumed. The agreement between their calculated  $r$  for the tonoplast and Blinks' value of  $r$  for whole cells is thus probably fortuitous. If Blinks' cells had been measured in MacRobbie and Dainty's bathing medium, their resistances would have been much lower.

The marked discrepancy between the calculated  $r$  from ion fluxes and measured values of  $r$  for cells (of which the tonoplast  $r$  is a small part) must have some other explanation. This is discussed by Hope and Walker (1960) and will be the subject of further work.

A consistent picture, however, seems to emerge in which the plasmalemma is the site of the resting potential and resistance, and, as Findlay (1959) has shown, of the action potential. It is clear, since the resistance of the cell falls greatly during the action potential, that it is the resistance of the plasmalemma which falls. This is not to say that the tonoplast plays no part in the action potential; simply that there is no evidence of this at the moment.

#### V. ACKNOWLEDGMENTS

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## APPENDIX I

## DERIVATION OF EQUATION (2)

It is assumed that the cell is a finite length of "leaky cable" immersed in a perfectly conducting medium. End effects are neglected. The resistance measured is that between the medium and a point on the axis of the cell, half way along its length.

Symbols:

- $R$  is the measured resistance ( $\Omega$ ),  
 $r$  is the surface resistance of the cell ( $\Omega \text{ cm}^2$ ),  
 $\rho$  is the resistivity of the interior of the cell ( $\Omega \text{ cm}$ ),  
 $L$  is the length of the cell (cm),  
 $D$  is its diameter (cm).

We may write

$$\lambda = \frac{1}{2} \sqrt{Dr/\rho},$$

where  $\lambda$  is a characteristic length, and

$$R_L = 4\rho L/\pi D^2,$$

where  $R_L$  is the longitudinal resistance of the cell interior, measured from one end to the other.

A conventional treatment of the problem (cf. Cole and Hodgkin 1938) leads to the following solution for  $R$  in terms of  $r$ :

$$R = (r/\pi DL) (L/2\lambda) \coth (L/2\lambda). \dots\dots\dots(3)$$

As expected, as  $L$  becomes  $\ll \lambda$ ,  $R \rightarrow r/\pi DL$ . Now in practice we measure  $R$  and wish to know  $r$ —i.e.  $\lambda$  is also unknown. A convenient approximation is

$$\coth x = (1/x) + (x/3) \quad (\text{for } 0 < x < 2).$$

Equation (3) then becomes

$$R = (r/\pi DL) + (R_L/12) \quad (\text{for } 0 < L < 4\lambda).$$

This is used in the form

$$r = \pi DL[R - (R_L/12)] \quad (\text{for } 0 < (R_L/12) < (R/2)).$$

This treatment neglects the external resistance, which in practice is not zero. However, the effect of the external resistance is not easy to take into account except in special cases. In general, a non-zero external resistance may increase or reduce the correction term  $(R_L/12)$ . If for equal elements of the cell surface the external resistance is equal, its effect is to make the current distribution over the cell surface more uniform, and to reduce the correction term. In the present experiments the external resistance was low compared with either  $R$  or  $R_L$ . It is considered that in this case it will not significantly alter the current distribution or the correction term.

## APPENDIX II

## TREATMENT OF BRIGGS'S MODEL

The model proposed by Briggs (1957) is one in which the cytoplasm is represented by a Donnan system in equilibrium with the external medium. It has a high concentration of fixed negative ions. The only diffusion barrier is at the tonoplast, which is permeable to anions only. The treatment below assumes that there are present only one species each of positive and negative diffusible univalent ions. We will treat the model as one-dimensional. The tonoplast will be supposed to be the plane  $x = 0$ , and the outer surface of the cytoplasm to be the plane  $x = a$ .

Then at any plane the following two equations hold:

$$\phi_{\pm} = -\frac{u_{\pm}kT}{e} \cdot \frac{\partial N_{\pm}}{\partial x} \mp N_{\pm}u_{\pm}\frac{\partial E}{\partial x}, \quad \dots\dots\dots(4)$$

where

$\phi_{\pm}$  is the net flux of positive (negative) ions (in ions/cm<sup>2</sup> sec),

$u_{\pm}$  is the electric mobility of the positive (negative) ions,

$N_{\pm}$  is the concentration of the positive (negative) ions (in ions/cm<sup>3</sup>),

$E$  is the electric potential, and

$k$ ,  $T$ , and  $e$  have their usual significance;\* and, since electric neutrality is preserved,

$$N_+ = N_- + N_A, \quad \dots\dots\dots(5)$$

where  $N_A$  is the concentration of fixed negative ions.

We will consider the apparent electric conductivity of the cytoplasm

(a) immediately the current is applied (before polarization), and

(b) when a steady state has been reached (when polarization is complete).

## (a) Before Polarization

In this case  $\partial N_{\pm}/\partial x = 0$  between  $x = 0$  and  $x = a$ , and  $J/e = \phi_+ - \phi_-$ , where  $J$  is the current density. Then

$$\frac{J}{e} = -\frac{\partial E}{\partial x}(N_+u_+ + N_-u_-). \quad \dots\dots\dots(6)$$

The cytoplasm conductivity is given by  $-J\left(\frac{\partial E}{\partial x}\right)^{-1}$ , and

$$-J\left(\frac{\partial E}{\partial x}\right)^{-1} = e(N_+u_+ + N_-u_-). \quad \dots\dots\dots(7)$$

This is clearly high since in the Donnan cytoplasm  $N_+ \cong N_A$ , which is assumed to be high.

\* In this equation  $ukT/e$  is written for the diffusion coefficient of a univalent ion.

(b) *After Polarization is Complete*

In this case  $\partial N_{\pm}/\partial t = 0$  for all values of  $x$ ; i.e.  $\partial\phi_{\pm}/\partial x = 0$ , and the  $\phi$ 's are constant. Now at  $x = 0$ ,  $\phi_+ = 0$  (the tonoplast is impermeable to cations). Hence  $\phi_+ = 0$  everywhere; and

$$-\frac{kT}{e} \frac{\partial N_+}{\partial x} - N_+ \frac{\partial E}{\partial x} = 0, \dots\dots\dots(8)$$

and

$$-\frac{kT}{e} \frac{\partial N_-}{\partial x} + N_- \frac{\partial E}{\partial x} = -\frac{J}{eu_-}, \dots\dots\dots(9)$$

Further, between  $x = 0$  and  $x = a$ ,

$$dN_- = dN_+. \dots\dots\dots(10)$$

This leads to

$$-J\left(\frac{\partial E}{\partial x}\right)^{-1} = eu_-(N_+ + N_-). \dots\dots\dots(11)$$

Again the cytoplasm conductivity is high, with a value appropriate to its high cation concentration, provided that  $u_-$  is not too small compared with  $u_+$ .

Briggs had claimed that the cytoplasm conductivity would be low, corresponding to the low value of  $N_-$ . It is clear that this claim is due to the erroneous application of verbal reasoning to the model. The model proposed, then, does not explain the present results, and a model including a plasmalemma is necessary.

# THE TRANSPORT OF CARBOHYDRATES IN AUSTRALIAN BRACKEN

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## Summary

Radioactive carbon dioxide was fed to bracken fronds and the form in which the labelled products of photosynthesis were moved into the rhizome and to growing buds determined as sucrose. It was shown that the products moving away from one frond were severely limited in their spread in the vascular system of the rhizome, being confined to part of the phloem of two of the thirty or so bundles in the neighbourhood of the frond, but spreading into more of the bundles in more distant parts of the rhizome.

## I. INTRODUCTION

In studies of the movement of systemic weedkillers in bracken, it became important to know whether the carbohydrate-transporting system was similar to that in higher plants, and in particular whether the transported sugar was sucrose. Previous work on the translocation of sugars has covered a wide range of plants: yam (Mason and Lewin 1926), cotton (Mason and Maskell 1928), *Pelargonium* (Schumacher 1930; Willenbrink 1957), palm (Tammes 1933), cucumber (Cooil 1941; Crafts and Lorenz 1944), sausage-tree (Bignoniaceae) (Clements 1940), maize (Loomis 1945), bean (Vernon and Aronoff 1952), *Quercus* and *Robinia* (Ziegler 1956), *Heracleum* (Ziegler 1958), and grapevine (Swanson and El-Shishiny 1958) but we are not aware of any translocation studies on ferns. In all plants studied, sucrose was the principal or the only sugar translocated, though traces of glucose and fructose were found in the grapevine, and Zimmerman (1957) showed that other sugars such as stachyose and raffinose could be transported in the phloem of *Fraxinus americana*. The present study was initiated as part of a programme to provide a background of information on the physiology of the Australian bracken.

## II. METHODS

### (a) Culture of Bracken

Spores were collected from wild plants growing in Victoria, sterilized for 10 min in 0.1 per cent. sodium hypochlorite solution, washed with sterile water, and sprayed on sterile sand wet with the nutrient solution of Schwabe (1951) in covered crystallizing dishes. Fertilization of the prothalli was carried out by adding a slight excess of water, and the young sporophytes were planted out in boxes of soil in a glass-house. The fronds of the plants used were 25–30 cm long from the insertion on the rhizome and had been growing in the glass-house for about eight months.

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*(b) Application of Labelled Carbon*

For each experiment a system was chosen where a mature frond was feeding into a young, actively growing frond, and ending in a rhizome apex beyond the growing frond, as shown in Figure 1. The path of the rhizome was traced out as accurately as possible without disturbing it or the roots, and the chosen system isolated by cutting the rhizome some distance back from the mature frond. A length of about 9 cm at the end of the mature frond was enclosed in a slit-stoppered conical flask containing about 1 mg of  $\text{Ba}^{14}\text{CO}_3$  ( $5 \mu\text{c}$ ) and a few drops of 80 per cent. lactic acid in a side-arm. The best results were obtained leaving the flask over the frond



Fig. 1.—The system used to study movement of labelled assimilate from a mature frond into a young frond and the rhizome apex. In some experiments the gas chamber covered the whole of the mature frond.

for a period of 6 hr under good daylight photosynthetic conditions. A relatively large dose of carbon dioxide (about 50 mg  $\text{Ba}^{14}\text{CO}_3$ ,  $1-2 \mu\text{c}$ ) for a period of 3 hr was also satisfactory. If the area of application was smaller, and in an experiment in which the application flask was removed after a period of 2 hr, labelled carbon was not detectable in the extracts from the apex and young frond.

*(c) Radioautography of the Whole Plant System*

The plant was washed free of soil, cut into pieces to prevent migration of labelled substances on drying, dried between several changes of blotting paper for

a week, and exposed to X-ray film for a further week. The developed film showed the gross distribution of labelled carbon in the plant at the time of harvest.

(d) *Radioautography of Sections of the Rhizome*

The whole plant was frozen with dry ice: hand-sections were made of rhizome and rachis with a cold razor-blade, and the sections placed on dry ice over phosphorus pentoxide in an evacuated desiccator. Two or 3 days of continuous evacuation of the desiccator was found sufficient to dry sections about 1 mm thick. The dried sections were exposed to X-ray film for periods depending on the dose of  $^{14}\text{CO}_2$  (usually at least 4 days).

(e) *Extraction of Labelled Substances from Bracken*

The experimental system was cut into the required fractions, usually the following: application area, rachis of application frond, rhizome (one or more parts), young frond, and rhizome apex. Tests showed that no radioactivity was detectable in the pinnae of the mature frond below those of the application area. Each piece was weighed and blended in hot 70 per cent. ethanol and the extract filtered and washed through the filter with more 70 per cent. ethanol. Tests in which radiosucrose was added to the tissue before blending showed that hydrolysis occurred overnight at room temperature, and extracts were stored at  $-20^\circ\text{C}$ . Hydrolysis was presumably due to the acidity of the extract and it is possible that resin deionization would stabilize the extract. Bracken tissue contains a very large amount of mucilage and other ethanol-soluble substances that we found impossible to remove without loss of about 20 per cent. of the radiosucrose by any of the methods suggested by Bell (1955). Methanol was tried as extracting solvent, but the extract contained larger quantities of substances that interfered with later processes. To ensure that no radioactive sugars were lost, therefore, the whole ethanolic extract of about 50 ml was spotted drop by drop on to chromatographic paper.

(f) *Chromatography of Extracts*

The ethanolic extract was run slowly from a dropping-funnel on to the starting line of a 1-in. wide strip of chromatographic paper (Whatman No. 3, acid-washed) and a jet of warm air was directed on the underside of the paper. If the air was too hot, test sucrose spotted in this way was found to be retained at the starting line in subsequent chromatography, presumably because it charred. Spotting was carried out with the air stream cool enough to avoid this. Chromatograms were run with the descending eluent ethyl acetate-pyridine-water (80 : 20 : 10 v/v) for 16–20 hr concurrently with a number of test sugars. The test strips were developed with aniline-hydrogen oxalate-resorcinol reagent (Zimmerman 1957). Radioactive spots were located on the paper strips by scanning with an automatic machine yielding a trace proportional to the specific activity at all points along the paper. The area under each peak of the trace was proportional to the amount of radiocarbon in the spot. A typical trace of a chromatogram is shown in Figure 2.

Tests in which the solvent front was still on the paper showed that nothing radioactive ran beyond fructose. After the initial run, chromatograms were scanned

for radioactivity and then if necessary the radioactive areas were eluted, sometimes further diluted, and run again. This procedure was found useful for purifying the extract from unwanted substances and would often resolve an area of bad trailing into sharp spots.

### III. RESULTS

#### (a) Radioautography

Plate 1, Figures 1 and 2, shows the spread of labelled carbon in 6 hr from a small application on the mature frond. Plate 1, Figures 3 and 4, shows the wider spread in a similar system after 24 hr. It will be noted that there is little movement into the untreated pinnae of a mature frond, and what little radiocarbon enters is soon exported again. Large amounts of radiocarbon accumulate in a growing frond after 24 hr, and even after 6 hr there is a trace shown on the X-ray film.

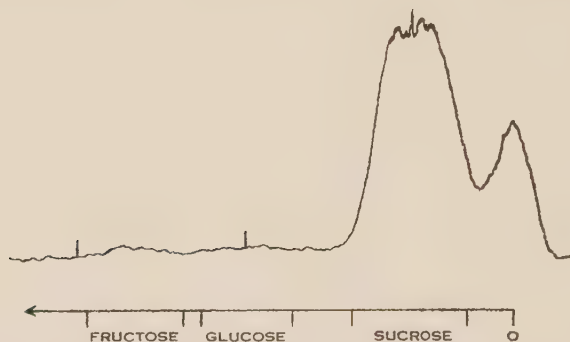


Fig. 2.—Trace produced in the automatic chromatogram scanner of the distribution of labelled carbon along a chromatogram of the extract of a rhizome apex. Both spots represent sucrose. The large one has the  $R_F$  of sucrose; the smaller one has been retained at the origin, but can be shown to be sucrose (as explained in the text).

Plates 2 and 3 show how the labelled carbon is distributed within a rhizome. Radioactive carbon dioxide was fed to the whole of the mature frond to avoid any possible confusion if different parts of the frond were connected to different parts of the vascular system of the rhizome, and radioautographs prepared of the sectioned rhizome after 6 hr. Plate 2 shows the section about 9 cm from the insertion of the frond. Near the application frond, the labelled products of photosynthesis appear only in a particular pair of bundles in the rhizome and are further localized in part of the peripheral phloem of these. The unexpected smallness of the area of the rhizome carrying labelled photosynthate explains the difficulty experienced in extracting labelled substances from the rhizome, where the preponderating bulk of tissue not carrying labelled carbon contributes unwanted substances that upset spotting and chromatography. Plate 3 shows the distribution of labelled substances in a part of the rhizome about 30 cm from the application frond. The anastomoses of the bundles have allowed exchange of labelled material between individual bundles

of the inner ring, and all bundles of this ring are now carrying carbohydrate derived from the single leaf treated, but still only one-sided arcs of the phloem of the bundles are involved in the transport.

### *(b) Nature of the Translocated Substance*

For reasons which were made clear by the radioautographs of rhizome sections, it is difficult to get a satisfactory characterization of the labelled substances present in such small proportion in that bulky organ. Clearer characterization may be obtained from the sinks to which the labelled photosynthate is moving and where it is accumulating—the young frond and the apex. Extracts from these two regions, when due precautions were taken to avoid breakdown, showed only one labelled substance soluble in 70 per cent. ethanol, namely sucrose. Figure 2 shows the scanning of a chromatogram from the rhizome apex. The solvent front has run far beyond the end of the paper, but no radioactive spots were present which ran beyond fructose.

To verify that the main spot in the extracts was sucrose, the spot was eluted, hydrolysed with 0.01N HCl for 20 min at 100°C, and the hydrolysed spot re-run after deionization with resin. The original spot had vanished and been replaced by two equal spots running in the same positions as glucose and fructose, both in the original solvent, and in *n*-butanol-ethanol-water (5 : 1 : 4 v/v).

In some extracts (see Fig. 2) a radioactive spot remained at the origin. This was an artefact produced in spotting and it was shown to be sucrose bound to the paper, as follows: (1) the starting area was smaller and the area of the sucrose spot was larger, when the chromatogram was run in *n*-butanol-ethanol-water (4 : 1 : 5 v/v), as compared with the same chromatogram run in ethyl acetate-pyridine-water; (2) the additional spot at the origin was formed with test-labelled sucrose spotted from a large volume of 70 per cent. ethanol if the temperature of the air stream was too high; (3) a single sucrose spot was obtained when the spot at the origin, produced either from test sucrose or from plant extracts, was eluted with water and spotted at a lower temperature. The paper from either test sucrose or plant extract, after elution with water, retained a small amount of radioactivity which was the same percentage of the original spot in both.

The rhizome extracts, though less convenient to work with, have not given results different from those obtained from the apices and young fronds. The chromatograms were less clear, but only sucrose was indicated. Labelled glucose and fructose were absent if proper precautions were taken.

## IV. DISCUSSION

The translocation system in bracken is seen to be like that in nearly all other species of plant studied in that the sugar transported is sucrose. That this should be so in a plant differing so widely in systematic position and in detailed structure, particularly in phloem structure, from all other plants so far studied argues some very special relation between the properties of this molecule and the mechanism of translocation. Further, the resistance of bracken to systemic weedkillers probably does not depend on any peculiarity of the translocation mechanism in the plant. Other plants are



able to carry systemic weedkillers along with translocated sucrose in the phloem, and it is suggested that the reasons why these compounds are not translocated in bracken be sought in some other stage of the process: difficulty of entry into the translocation system, protein binding of the hormone molecule, or some other inactivation mechanism.

The detailed radioautographs of the location of the sucrose in the rhizome appeal to us as the most interesting feature of the work, for, so far as we know, pictures of this resolution have not been produced before for the localization of the translocated sucrose. The fact that labelled sucrose can travel some centimetres in the rhizome, localized not merely to one or two bundles but to part only of the phloem of the bundles, undermines a number of assumptions which physiologists have taken over untested from the anatomists. It has been tacitly assumed that an anastomosis of bundles implies a ready exchange of substances carried in the bundles; that a continuity of vascular path between two points is sufficient to imply the ready translocation of foodstuffs between the two points; and conversely, that an absence of vascular connection rules out any possibility of such transfer. The pictures here presented show that we may make no deductions about the path of translocation from a study of the vascular paths, but must determine by experiment where the sugar goes.

#### V. ACKNOWLEDGMENT

We should like to record our thanks to the Directors of I.C.I.A.N.Z. for permission to publish this work.

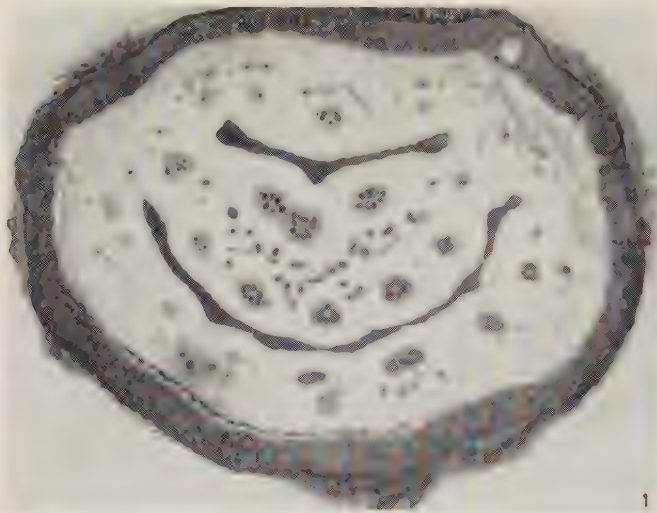
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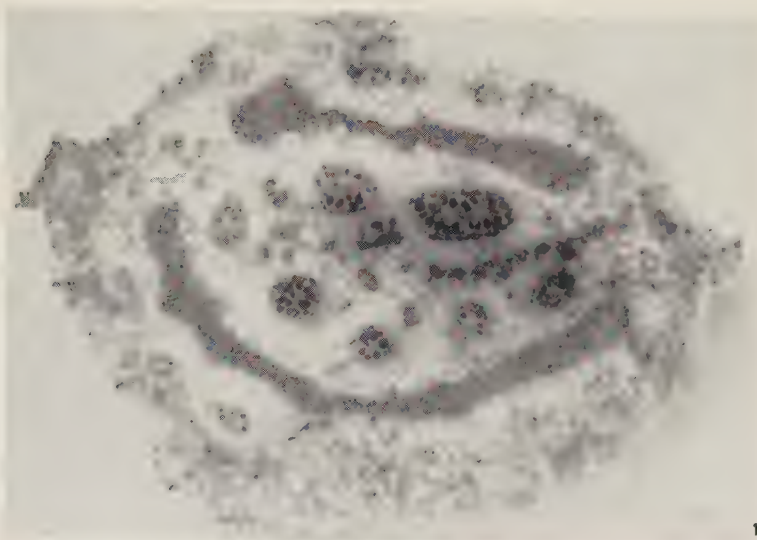
TRANSLOCATION IN BRACKEN



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## EXPLANATION OF PLATES 1–3

## PLATE 1

- Figs. 1 and 2.—Distribution of labelled photosynthate in the bracken system 6 hr after application to a pinna of the mature frond: plant (Fig. 1); radioautograph (Fig. 2). Labelled carbon is just detectable in the uncurling young frond.
- Figs. 3 and 4.—Distribution of labelled carbon 24 hr after application to a single pinna of the mature frond: plant (Fig. 3); radioautograph (Fig. 4). The application frond is empty of labelled carbon except for the immediate neighbourhood of the treated pinna. The young frond and rhizome are heavily labelled.

## PLATE 2

- Figs. 1–3.—Distribution of labelled photosynthate in the rhizome near the application frond: tissue (Fig. 1); radioautograph (Fig. 2); the two figures superimposed (Fig. 3). The sucrose is moving in part of the phloem of two vascular bundles only, though faint traces are perceptible in five others.

## PLATE 3

- Figs. 1 and 2.—Distribution of labelled sucrose in the bundles of the rhizome remote from the application frond: tissue and radioautograph (Fig. 1); radioautograph (Fig. 2). Anastomoses of the bundles have permitted the spread of labelled sucrose to most of the members of the inner ring.

# THE BREAKDOWN OF 2,4-DICHLOROPHENOXYACETIC ACID IN SHOOTS AND ROOTS

By M. J. CANNY\* and KATALIN MARKUS†

[Manuscript received March 31, 1960]

## Summary

Measurements have been made of the rate of evolution of labelled carbon dioxide from shoots and from roots of tick beans which have been treated on one leaflet with a small dose of labelled 2,4-dichlorophenoxyacetic acid (2,4-D). The corresponding breakdown of 2,4-D in root and shoot has been calculated. The carbon dioxide evolved from the roots was consistently more radioactive than that from the shoot suggesting that 2,4-D is more rapidly broken down in the former and the total loss of labelled carbon in 4 days corresponded to about 5 per cent. of the applied dose of growth substance. 2,4-D was not readily extractable from the roots at the conclusion of the respiration run and the labelled carbon was shown to be present there in many compounds that were not 2,4-D. It is concluded that though part of the side-chain was rapidly lost from the plant as carbon dioxide from the roots, this is not a main pathway involved in the inactivation of this growth substance. The evidence from intact plants points strongly to most of the breakdown occurring in the roots. With cut tissue, similar radioactive assays and bioassays did not reveal a greater intrinsic capacity of the roots for 2,4-D breakdown.

## I. INTRODUCTION

The many reports of the destruction of 2,4-dichlorophenoxyacetic acid (2,4-D) in plants and its conversion to substances that are not growth regulators (Holley, Boyle, and Hand 1950; Fang *et al.* 1951; Fawcett, Ingram, and Wain 1952; Holley 1952; Jaworski and Butts 1952; Jaworski, Fang, and Freed 1955; Hay and Thimann 1956*a*, 1956*b*; Weintraub *et al.* 1952, 1959) have not suggested that the process is localized in any part of the plant. 2,4-D is reported to be carried with the translocated sugars moving therewith to the apex, young leaves, and throughout the stem (e.g. Fang *et al.* 1951; Hay and Thimann 1956*b*), and the breakdown is presumed to take place generally through these organs. As a herbicide it is ineffective on many plants with deep root systems, causing distortion and death of the shoot, but permitting regrowth from the root-stock. This fact received an apparent explanation from the work of Hay and Thimann (1956*b*) who record that transport of 2,4-D "ceases at about the middle of the hypocotyl, none entering the roots at any time". Their method of assay was a biological one that was specific for 2,4-D but would not reveal any of its breakdown products, in contrast to the methods of other workers who have used <sup>14</sup>C-labelled acid. When labelled 2,4-D is applied to a plant of *Vicia* or *Phaseolus* (the two species commonly used), labelled carbon appears in the roots, but slowly compared with the speed of dissemination in the shoot. Initially it is found only in the upper parts of the roots, although the main stream

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of sugar transport is to the root tips (see radioautographs in Plate 1). If, therefore, 2,4-D does not appear in the roots, it is of interest to know whether it is not transported there, or is broken down on arrival, the traces of radiocarbon being due to breakdown products. It is also possible that only a small amount of radiocarbon is observed in the roots because much of it is lost as carbon dioxide. These with several other considerations have prompted us to compare both the breakdown of 2,4-D in shoot and root tissue of the tick bean (*Vicia faba* var. *minor*) and also the quantity broken down in the shoot and root of an intact plant as measured by the evolution of labelled carbon dioxide from the two halves of the plant when labelled 2,4-D is applied on a leaf. Acids labelled in both methylene and carboxyl groups have been used to compare the fates of these two carbon atoms.

## II. METHODS

### (a) *Tissue Respiration*

Pieces of tissue about 2 cm long were injected in a vacuum desiccator with an aqueous solution of the ethanolamine salt of 2,4-D at concentrations around 10 mg/l of 2,4-D (acid equivalent). Above this concentration there is a danger of toxic effects. The injected tissue was washed three times with distilled water, blotted, and placed in a microrespirometer consisting of a 50-ml beaker and a small bell-jar. Air was passed successively through soda-lime, lime water, the respirometer, and 15 ml of 0.2N NaOH in a Pettenkofer tube. With 1 or 2 g of tissue in the respirometer, a convenient quantity of carbonate was produced in 6 hr. No temperature control was available, but, since we were interested only in the comparative production of labelled CO<sub>2</sub> by two different tissues and the respirometers stood side by side, changes in temperature can be neglected. The tissue was weighed before and after injection. At various intervals during the measurement of respiration, samples could be withdrawn, extracted with hot 80 per cent. ethanol, and <sup>14</sup>C-containing substances estimated by chromatography and scanning.

### (b) *Respiration of the Intact Plant*

The tick bean was germinated in a small pot and, when the shoot had just emerged from the soil, a plate of glass with a hole in the centre was placed over it so that the plant developed its shoot system above and its root system below the glass. The glass was transparent or opaque according as it was desired to measure respiration of roots in the light or dark.

When the plant had attained a convenient size the pot was removed and the roots washed free of soil. The hypocotyl was sealed in the glass plate with cocoa-butter, and two bell-jars fixed to the plate with lanolin, one enclosing the root system and one the shoot. Wet blotting-paper was pressed to part of the wall inside each bell to keep the humidity high. When the root respiration was to be measured in the dark, the lower bell-jar was darkened with insulating tape as shown in Figure 1. Moist air or moist air free of CO<sub>2</sub> could be supplied independently to either half of the plant, and the outlets led into separate Pettenkofer tubes containing NaOH.



A leaflet of the shoot was treated with the dose of 2,4-D acid, labelled in the methylene or carboxyl group, dissolved in about 5  $\mu$ l of 90 per cent. ethanol containing 1 per cent. "Tween 20". During the day the shoot was supplied with daylight and air while  $\text{CO}_2$  was collected from the roots with a current of  $\text{CO}_2$ -free



Fig. 1.—Tick bean with shoot and root enclosed in separate respiration chambers allowing different gas streams to be fed to the two halves, and samples of the respired carbon dioxide from the two halves to be taken independently. The root system is darkened; the shoot may be exposed to light, and air supplied to it to enhance the transport of carbohydrate and 2,4-D to the roots.

air. At night the whole plant was darkened and the  $\text{CO}_2$  output from both shoot and root collected with a current of  $\text{CO}_2$ -free air. By allowing the shoot to carry on sugar synthesis in the daytime the transport of 2,4-D to the roots was greatly

increased as measured by the output of labelled  $\text{CO}_2$ . After 5–7 days of measurement, the plant was either cut, dried, and placed on X-ray film to obtain a radioautograph of the final distribution of labelled carbon, or various parts of it were blended in hot 80 per cent. ethanol for more detailed chemical examination.

### (c) Carbon-14 Assay

The carbonate was precipitated with 30 ml of 0.05M  $\text{BaCl}_2$  and the precipitate centrifuged off, washed, and deposited for counting on a tared filter disk as a plate of known area. The unreacted  $\text{NaOH}$  was titrated against standard  $\text{HCl}$ , and the total  $\text{BaCO}_3$  produced was calculated. All  $\text{BaCO}_3$  precipitates were counted with the same geometry relative to a thin-window Geiger tube (type EW3H) for at least 10,000 counts, and corrected to infinite thickness, and for drift and background.

### (d) Calibration of the Radioactivity of the Carbonate in Terms of 2,4-D Breakdown

A known mass of each of the two labelled 2,4-D acids (*c.* 20  $\mu\text{g}$ ) was diluted with 10 mg of phenoxyacetic acid (not chlorine substituted, lest the chlorine should interfere with the determination of  $\text{CO}_2$ ) and burnt under vacuum in a closed system with a Van Slyke–Folch mixture. The  $\text{CO}_2$  produced was absorbed in standard  $\text{NaOH}$ , precipitated with  $\text{BaCl}_2$ , and the precipitates mounted and counted at infinite thickness in the same system as the tissue precipitates. The unreacted  $\text{NaOH}$  was titrated and the total carbonate calculated. Ten determinations were made for each acid and the mean used to calculate the quantities of 2,4-D broken down that correspond to a particular richness of labelling of the  $\text{BaCO}_3$  formed. Thus:

$$\begin{aligned} \frac{\text{2,4-D broken down}}{\text{2,4-D burnt}} &= \frac{\text{total counts from gas}}{\text{total counts from combustion}} \\ &= \frac{\text{mass of ppt. from gas} \times \text{specific activity}}{\text{mass of combustion ppt.} \times \text{specific activity}} \\ &= \frac{\text{mass of ppt. from gas} \times K \times \text{counts at infinite thickness}}{\text{mass of combustion ppt.} \times K \times \text{counts at infinite thickness}}, \end{aligned}$$

where  $K$  depends on the counting arrangement, but is the same for all samples. The calculation of the 2,4-D breakdown corresponding to a particular richness of carbonate labelling therefore resolves itself into

$$\text{2,4-D broken down} = \text{mass of tissue ppt.} \times \text{counts at infinite thickness} \times A,$$

where  $A$  is a composite constant obtained by counting the burnt samples in the same counter. It will be noticed that the measurement is unaffected by diluting, inactive carbonate, which decreases the counts at infinite thickness in the same proportion as it increases the mass of the precipitate. This has great practical importance for it means that neither variations in the production of unlabelled  $\text{CO}_2$  by the tissue, nor leaks in the gas system admitting aerial  $\text{CO}_2$  to the  $\text{NaOH}$ , need be taken into account in calculating the 2,4-D breakdown.

### (e) Chromatography

Extracts of tissue containing 2,4-D were chromatographed on 1-in. wide strips of acid-washed Whatman No. 3 or 3 MM paper in *n*-butanol–ammonia–water

(10 : 1 : 1 v/v) for 5 hr. Chromatograms were scanned in an automatic machine yielding a trace proportional to the specific activity at all points along the paper. The quantity of labelled carbon in each peak on the trace was measured by planimetering the area under the curve.

#### (f) Bioassay

Quantities of 2,4-D in some extracts were estimated by the leaf-repression method of Brown and Weintraub (1950) which is specific for this type of growth regulator, and little responsive to indolylacetic acid. The cultivar of *Phaseolus* used is not Black Valentine, as suggested by these authors, but *Epicure*. Substances to be assayed are applied, in 5- $\mu$ l drops of 90 per cent. ethanol containing 1 per cent. "Tween 20", to the young bud between the first foliage leaves, and the percentage repression of mass of the first trifoliate leaves that develop in 7-14 days is measured relative to those treated with solvent only. There is a linear relationship between the percentage repression and the logarithm of the dose of growth substance. This assay is a standard one for growth activity in this Laboratory and the response curve of the beans used to different doses of 2,4-D acid has been measured many times. It is therefore possible to use the test in the reverse sense, and measure quantities of 2,4-D down to  $10^{-8}$  g by the percentage repression of leaf growth of the beans.

### III. RESULTS

#### (a) Respiration of Cut Tissue

Because of the extreme sensitivity of plant tissue to 2,4-D, the quantity which could be injected into tissue had to be kept low. This is because of its effect, not on respiration which work in this Laboratory has shown to be negligible up to 1000 p.p.m. (O'Brien, unpublished data 1959), but on some other metabolic system, and, indeed, higher concentrations than about 10 p.p.m. caused inhibition of the breakdown of 2,4-D which was being measured. Since the available radioactive 2,4-D has a low specific radioactivity, the small amounts used meant that the radioactivity of the treated tissue was low, and that of the evolved  $\text{CO}_2$  even lower. When this  $\text{CO}_2$  was precipitated as  $\text{BaCO}_3$ , the specific radioactivity of the precipitate was little above background, making assay of it a matter for lengthy counting.

Breakdown of 2,4-D injected into root and shoot pieces was nevertheless detectable in all tissues treated with from 0.5 to 15 p.p.m. 2,4-D acid equivalent at rates varying from 0.1 to 1  $\mu\text{mg}$  of 2,4-D/g fresh wt. of tissue/hr. For example, Table 1 contains data for the respiration and 2,4-D breakdown as measured by evolution of  $^{14}\text{CO}_2$  by roots treated with a solution of 5 p.p.m. of carboxyl-labelled 2,4-D, one chamber being kept in the dark and the other in the light. There is no significant difference between the reactions in the light and dark, and the rate of breakdown is steady at about 0.3  $\mu\text{mg}$ /g fresh wt./hr. The slowly falling rate of  $\text{CO}_2$  production reflects the loss of reserves and general damage to the tissue, and the effect of variation of temperature from day to night is seen to be more marked on the respiratory process than on the breakdown of 2,4-D. Because these measurements were made at the extreme limits of detection, they were not of sufficient

accuracy to reveal any consistent difference between the rates of breakdown in shoot and root tissue, and attention was turned to the comparative outputs of labelled  $\text{CO}_2$  from root and shoot in the intact plant.

(b) *Respiration of Intact Plants*

Single tick bean plants were treated with a dose of from 2 to 20  $\mu\text{g}$  of 2,4-D on a leaflet and the respiratory  $\text{CO}_2$  from shoot and roots sampled separately and assayed for radioactivity as described above. The roots were generally darkened and maintained for the duration of the experiment in  $\text{CO}_2$ -free air so that continuous

TABLE 1  
RESPIRATION AND BREAKDOWN OF 2,4-D IN CUT ROOT TISSUE UNDER BOTH LIGHT AND DARK CONDITIONS  
5 mg/l of carboxyl-labelled 2,4-D acid equivalent injected as ethanolamine salt. Respiration chambers side by side under the same temperature regime, one in continuous light, one in continuous darkness

Time to Middle of Sampling Period (hr)	Duration of Sampling Period (hr)	CO <sub>2</sub> Production ( $\mu\text{l/g}$ fresh wt./hr)		2,4-D Breakdown ( $\mu\text{mg/g}$ fresh wt./hr)	
		Dark	Light	Dark	Light
8	17.0	—	145	—	0.21
20	7.2	108	172	0.03	0.23
33	17.7	98	95	0.26	0.23
46	7.5	132	127	0.29	0.51
58	17.5	65	84	0.26	0.37
70	7.5	96	115	0.40	0.48
83	17.5	50	64	0.34	0.39

assay of the root  $\text{CO}_2$  was possible. To keep the plant healthy, it was necessary to supply the shoot with daylight and air in the daytime, which meant that the respiratory  $\text{CO}_2$  from the shoot could be sampled only at night. Most doses were large enough to cause marked growth curvatures of the stems.

Data from one such experiment are assembled in Table 2. A dose of 15  $\mu\text{g}$  of methylene-labelled 2,4-D was placed on a leaflet and the root  $\text{CO}_2$  was sampled continuously for 86 hr, and the shoot  $\text{CO}_2$  at night only during the same period. The  $\text{CO}_2$  production from the root in the first sampling period is high, presumably due to handling, but thereafter for the remainder of the experiment the rate falls only slowly and is higher in the day than at night. On a fresh weight basis within the one period, the rates of  $\text{CO}_2$  evolution from shoot and root are similar.

The 2,4-D breakdown calculated from the richness of labelling of the respired  $\text{CO}_2$  is given in two units for each sampling period: first as the total quantity in micrograms broken down in each region in the period, and second as the rate of breakdown in  $\mu\text{mg/g}$  fresh wt./hr. The second of these rates has been plotted in Figure 2 showing how the rate of breakdown in the roots is maintained at a level



TABLE 2  
RESPIRATION AND BREAKDOWN OF 2,4-D IN SHOOT AND ROOT OF INTACT TICK BEAN

15  $\mu\text{g}$  of methylene-labelled 2,4-D on leaflet at zero time. Shoot in light and air in the daytime (the short periods of the table), and in dark and  $\text{CO}_2$ -free air at night. Roots in continuous darkness and  $\text{CO}_2$ -free air. Mass of shoot system 1.52 g; mass of roots 2.56 g

Time to Middle of Sampling Period (hr)	Duration of Sampling Period (hr)	CO <sub>2</sub> Production ( $\mu\text{l}$ )		CO <sub>2</sub> Production ( $\mu\text{l/g}$ fresh wt./hr)		2,4-D Breakdown ( $\mu\text{mg}$ )		2,4-D Breakdown ( $\mu\text{mg/g}$ fresh wt./hr)	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
3	6.25	—	5110	—	320	—	0	—	0
14	16.5	4060	7280	162	172	25.3	35.1	1.01	0.83
26	7.4	—	4130	—	218	—	59.8	—	3.16
38	16.3	3330	5560	135	133	18.9	166	0.76	3.98
50	7.7	—	3150	—	160	—	123	—	6.25
62	16.3	2500	3935	101	94	25.9	145	1.05	3.48
74	7.7	—	2840	—	144	—	76.6	—	3.88
86	16.3	2160	4080	87	98	16.4	145	0.67	3.48
		Total				86.5	750.5		

much above that of the shoot for most of the 86 hr. At the foot of the column of 2,4-D breakdowns in Table 2, the sum of all the breakdowns in each region is given. The whole of the labelled  $\text{CO}_2$  output from both halves of the plant in 86 hr is equivalent to a loss of about 850  $\mu\text{mg}$ , which is 5.7 per cent. of the applied dose. If it is assumed that the shoot was breaking down 2,4-D at about the same rate during the days when it was not measured, the percentage will be something over 6, and the total breakdown in the roots something like seven times that in the shoot, in spite of the dose having been applied to the shoot and all the 2,4-D in the roots having to be translocated there. The rate of 2,4-D breakdown in the roots is apparently much less sensitive to change of temperature than the respiratory  $\text{CO}_2$  evolution since the rate is remarkably constant with the exception of one reading after the first 24 hr.

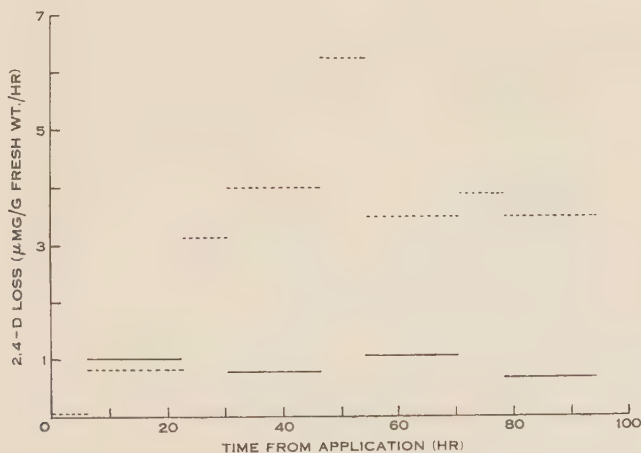


Fig. 2.—Rate of breakdown of 2,4-D in  $\mu\text{mg/g}$  fresh wt./hr plotted against time from application in the shoot (—) and root (---) as measured by the rate of evolution of  $^{14}\text{CO}_2$  from the two regions.

No difference was detectable in the rate of breakdown of 2,4-D labelled in the carboxyl position, although other workers have found this carbon atom to be the more labile (Weintraub *et al.* 1952). Table 3 shows the data of an experiment with the carboxyl-labelled acid in which the dose was 2.4  $\mu\text{g}$  and sampling was delayed for 2 days after the application. The rate of breakdown is reduced relative to that in Table 2 in the same ratio as the dose, and the total breakdown in 86 hr can be estimated as still about 6 per cent. of the applied dose. The rate of breakdown in the roots is again much higher than in the shoot.

### (c) *Labelled Carbon in the Plant at the End of the Respiration Run*

Radioautographs of experimental plants at the conclusion of the measurements of respiration and 2,4-D breakdown showed that nearly all the labelled carbon had moved from the shoot into the root, but, as in Plate 1, was present mainly in the upper part of the root system. Plate 2 shows the distribution of radiocarbon in the plant

TABLE 3

RESPIRATION AND BREAKDOWN OF 2,4-D IN SHOOT AND ROOT OF INTACT TICK BEAN

2.4  $\mu\text{g}$  of carboxyl-labelled 2,4-D on leaflet at zero time. Shoot in light and air in the daytime, and in dark and  $\text{CO}_2$  free air at night. Roots in continuous darkness and  $\text{CO}_2$ -free air. Fresh weight of shoot system 1.92 g; of roots 4.40 g. Sampling begun after 2 days

Time to Middle of Sampling Period (hr)	Duration of Sampling Period (hr)	CO <sub>2</sub> Production ( $\mu\text{l}$ )		CO <sub>2</sub> Production ( $\mu\text{l/g}$ fresh wt./hr)		2,4-D Breakdown ( $\mu\text{mg}$ )		2,4-D Breakdown ( $\mu\text{mg/g}$ fresh wt./hr)	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
48	7.6	—	5930	—	173	—	27.5	—	0.80
60	16.3	5000	6300	160	86	0	36.5	0	0.48
72	9.5	—	4780	—	112	—	26.0	—	0.61
84	14.5	4095	5510	178	85	2.4	34.1	0.09	0.52
96	7.7	—	4205	—	122	—	30.8	—	0.89
		Total 2.4				154.9			

that yielded the data of Table 2. Plants whose shoots were not supplied with air and light at intervals showed the opposite picture, all the radiocarbon remaining in the shoot, but did not yield any more  $^{14}\text{CO}_2$  from the shoot and none from the root. An ethanolic extract of the root system of a plant like that in Plate 2 when chromatographed in *n*-butanol-ammonia-water gave a broad, radioactive peak centred on  $R_F$  0.50. The  $R_F$  of 2,4-D acid in this solvent is about 0.70. This broad peak, when extracted with ether and re-run in the same solvent, gave a pink radioactive spot with  $R_F$  0.42 (36 per cent. of the original spot). If the paper remaining after ether extraction was further extracted with ethanol and this extract re-run, a single sharp spot at  $R_F$  0.55 was obtained (62 per cent. of the original spot); and a third extraction of the paper with water yielded three spots ( $R_F$ 's 0.15, 0.37, and 0.66) making in aggregate 9 per cent. of the original spot (total 107 per cent.). In the bioassay of the extract no growth activity was detectable, so that even the ether-soluble part of the extract is not 2,4-D. Clearly there has been extensive metabolism of the radioactive atoms from the side-chain of 2,4-D and only a small part of this has involved the loss of labelled  $\text{CO}_2$  from the plant. The absence of 2,4-D in the roots is in accord with the results of Hay and Thimann (1956b), but by far the larger part of this disappearance involves other processes than that which we have been measuring by the evolution of labelled  $\text{CO}_2$ .

Although the 2,4-D is moving from shoot to root, and both the quantity of 2,4-D in each region and the total quantity of ether-extractable 2,4-D are changing progressively with time, the rate of 2,4-D breakdown per gram fresh weight of each region remains fairly steady. This suggests that the chosen unit of rate of breakdown is of value for the kind of comparison between the regions here attempted, in spite of the fact that we have no clear idea where the breakdown is going on, or what is the concentration of 2,4-D at the site of breakdown.

(d) *Measurement of the Disappearance of 2,4-D in Root and Shoot Tissue by Bioassay*

Having shown that the loss of 2,4-D that resulted in direct evolution of part of the side-chain as  $\text{CO}_2$  was insufficient to account for the disappearance of the substance in the plant, it became of interest to find what else was happening to it, and whether this disappearance was more rapid in roots than in shoots. Since the bioassay of 2,4-D is both more sensitive, and more specific, than the radioactive assay, a measurement was attempted by this means of the disappearance of ether-extractable 2,4-D in shoot and root tissue.

About 15 g of each of the cut stem and root tissue from tick beans was weighed and injected (under vacuum) with an aqueous solution of 5 mg/l of 2,4-D acid, washed three times with distilled water, blotted, and again weighed. The tissues were stored in the dark in polythene bags. Samples (2 g) were taken of each tissue before injection and at intervals after injection, extracted with ether, and the ether extracts assayed for 2,4-D using the leaf-repression test. The blank value for untreated tissue was subtracted from each measurement. Ether-extractable 2,4-D as measured by this means disappears rapidly in both tissues as shown in Figure 3. The stem contains more intercellular spaces than the root, so the initial content of stem tissue is greater, and 2,4-D takes longer to disappear entirely, but the rates



of disappearance in both tissues are broadly the same in each experiment. The mean rate of disappearance in the long-term experiment is  $4.8 \mu\text{mg/g}$  fresh wt./hr in the stem and  $4.5 \mu\text{mg/g}$  fresh wt./hr in the roots, and in the short-term experiment, 2.8 and 3.6 of the same units in the stem and root respectively. These are in close agreement with the higher rates for breakdown in the roots measured by radioactive assay in the intact plant, but about 10 times those measured in cut tissue in the comparable experiment of Table 1. It will be noticed that the highest initial 2,4-D content ( $0.3 \mu\text{g/g}$  fresh wt.) is presumably much less than the local tissue content around a part of a plant treated with a dose of  $10 \mu\text{g}$  in  $c.5 \mu\text{l}$  alcohol, but we have no information about actual local tissue contents at the site of breakdown of the translocated 2,4-D. It is possible that a higher rate of disappearance obtains locally where there is higher concentration of 2,4-D, but, as mentioned earlier, injection of solutions stronger than about  $10 \text{ mg/l}$  causes a reduction in the evolution of  $^{14}\text{CO}_2$ , so the rates at higher concentrations are not accessible by the injection-bioassay type of measurement.

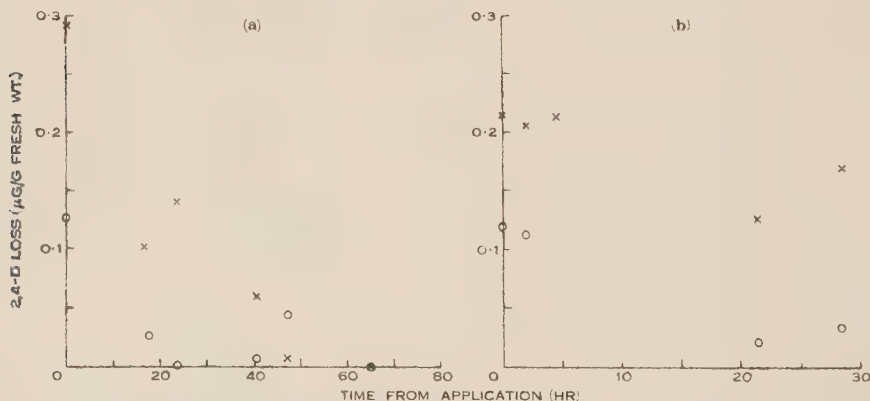


Fig. 3.—Disappearance of ether-extractable 2,4-D from tick bean shoots (x) and roots (o) as measured by bioassay: (a) results of an experiment lasting 80 hr; (b) the change in the first day after injection.

This disappearance of ether-extractable 2,4-D recalls the experiments of Holley, Boyle, and Hand (1950) and Holley (1952) who showed that radioactive 2,4-D was progressively bound into a water-soluble complex from which could be recovered by acid or alkaline hydrolysis, a radioactive substance very similar to 2,4-D, but separable from it in a counter-current apparatus. We have confirmed that the 2,4-D which disappears as outlined above when injected into tick bean root or shoot tissue, can be recovered quantitatively by acid or alkaline hydrolysis from aqueous extracts of the tissue, though we have not been able to show that the recovered substance is in any way different from 2,4-D. Aqueous extracts were made of the tissues at intervals after injection, hydrolysed with NaOH or HCl at  $100^{\circ}\text{C}$ , neutralized, and an ether extract of the hydrolysate assayed by means of the leaf-repression test. Growth activity was found corresponding to all the 2,4-D that

was not extractable by ether from the tissue. Radioactive growth substance recovered from the hydrolysed water extract of tissue treated with labelled 2,4-D could not be separated from known 2,4-D by chromatography in any of the solvents: *n*-butanol-ammonia-water (10 : 1 : 1 v/v), ethyl acetate-pyridine-water (80 : 20 : 10 v/v), or *n*-butanol-ethanol-water (5 : 1 : 4 v/v). A Craig counter-current apparatus was available, but the repetition of this lengthy assay was not attempted. We are prepared to believe that the recovered growth substance may be sufficiently different from 2,4-D to be separated from it by this means, but assert that it is sufficiently like 2,4-D to have the same effect in the leaf-repression assay, and the same  $R_F$  in the solvents enumerated.

TABLE 4

2,4-D RECOVERED FROM THE ROOT PROTEINS OF TICK BEANS AFTER APPLICATION OF 10  $\mu$ g OF 2,4-D ON A LEAFLET OF THE SHOOT  
2,4-D estimated by bioassay of the ether extract of hydrolysate of root proteins

Plants Harvested 2 Days after Treatment	2,4-D Recovered ( $\mu$ g)	Plants Harvested 4 Days after Treatment	2,4-D Recovered ( $\mu$ g)	Plants Harvested 7 Days after Treatment	2,4-D Recovered ( $\mu$ g)
No. 1	0	No. 4	10	No. 7	0
2	0	5	10	8	0
3	5	6	10	9	0

(e) *Fate of the 2,4-D in the Roots*

The evidence so far presented suggests that the 2,4-D which enters the roots in the translocation stream is very probably bound there in complexes with proteins, and it is reasonable to deduce that it could be recovered again from aqueous extracts of the roots by hydrolysis. This deduction was proved to be correct. Nine tick beans were treated simultaneously on leaflets with 10  $\mu$ g of 2,4-D in c. 5  $\mu$ l ethanol. Three plants were harvested at each of the following intervals after treatment: 2 days, 4 days, and 7 days. Aqueous extracts were made of the root system of each by grinding with dry ice and taking up in water. The filtered extracts were hydrolysed with 0.1N NaOH at 100°C for 5 hr, neutralized, and extracted with ether. The ether extracts were assayed for growth activity in the leaf-repression test and the results are expressed in terms of equivalent quantities of 2,4-D in Table 4. This crude experiment suffices to show that the statement of Hay and Thimann (1956b) that was recorded in the Introduction is not true. 2,4-D does enter the roots, though it is not extractable therefrom by ether. After 4 days it may be recovered from an aqueous extract of the roots in a form that is still growth-active, and this accounts for the larger part of the dose applied to the shoot. If it is changed as Holley claims, the change is so slight as to be negligible. After 7 days it has apparently all been broken down to the many substances found by chromatography at about this time.

## IV. DISCUSSION

The production of  $^{14}\text{CO}_2$  by tissue treated with labelled 2,4-D is unequivocal evidence of breakdown of the growth substance in the tissue, but it is not evidence of the direct production of  $\text{CO}_2$  in one or a few steps from the parent acid. Extensive transformations may have taken place between the known starting molecule and that which we measure, and we cannot argue that there has been direct decarboxylation of the carboxyl-labelled acid or  $\beta$ -oxidation of the methylene-labelled acid. Taking the whole set of results together, it seems more likely that the mechanism involves a loss of the acetic side-chain more or less intact, and its subsequent metabolism to  $\text{CO}_2$  for this would explain the equal  $^{14}\text{CO}_2$  evolution from carboxyl- and methylene-labelled acids.

As for the greater richness in labelling of  $\text{CO}_2$  from the roots, it might be objected that this was due to breakdown of labelled 2,4-D in the shoot to  $\text{CO}_2$ , followed by resynthesis of this in the light periods to carbohydrate, and export of  $^{14}\text{C}$ -sucrose to the roots where it would be respired to  $^{14}\text{CO}_2$ . However, this mechanism would require that the  $^{14}\text{CO}_2$  produced by the shoot in the dark and which cannot be refixed, should be comparable in quantity to that measured from the roots, the opposite of what is observed. Secondly, the radioautograph of the root system after measurement (Plate 2) is not that typical of the distribution of labelled photosynthate (Plate 1, Fig. 2), but shows a typical 2,4-D pattern.

Translocation of labelled carbon to the roots is clearly rapid, if less so than for carbohydrate, and probably the labelled carbon is still mostly in the form of 2,4-D. As is clear in Plate 1, the concentration of 2,4-D in the shoot is vastly greater than in the root for the first day or two, and, though we have been unable to demonstrate any greater intrinsic capacity of the cut roots for destroying 2,4-D, it is suggested that the higher output of  $^{14}\text{CO}_2$  by the roots is real evidence of a higher rate of breakdown there in the intact plant. That present in the shoot is sufficient to cause marked growth distortions of the stem and some of it suffers the little breakdown we have measured, but the sink for 2,4-D is the upper part of the root system where it suffers both binding to proteins and, later, extensive metabolism. It causes there much less in the way of growth effects in what is generally supposed a much more auxin-sensitive part, probably because it is more firmly bound than in the shoot.

The absence of labelled carbon in the root tips and recently elongated root which was shown in Plate 1, has now received partial explanation. The 2,4-D is not lost, but retained in the upper part of the root system bound to proteins and it may be recovered by hydrolysis in a growth-active form. Similar complexes can also be formed in the shoot, but for some reason are less permanent, for the labelled 2,4-D vanishes from there and appears bound in the upper roots between the second and fourth days after treatment of the leaflet (compare Plates 1 and 2). The reason why the translocated 2,4-D does not pass the upper roots is still obscure, but we may postulate either a rapid leakage from the transport system there, or a more tenacious binding to the root proteins. The latter seems more likely.

The analogy with endogenous auxin is close, since the indolylacetic acid produced by the shoot must be broken down before entering the much more sensitive



root system if it is not to upset the control of root growth by the auxin produced in the root apices, and high concentrations of auxin-inactivating enzymes have been found in the roots (Wagenknecht and Burris 1950; Tang and Bonner 1957). It is possible that the exogenous growth substance (2,4-D) is becoming involved in the same breakdown system, in spite of the fact that the experiments with cut tissue have failed to show the presence of a more active system in the roots; for the system may be of a type which would not be revealed by assay either of evolved  $^{14}\text{CO}_2$  or of ether-extractable 2,4-D. This introduces us to a second consideration.

There are two tenfold discrepancies in the measurements of rate of destruction of 2,4-D described above: in cut tissue the rate of disappearance of ether-extractable 2,4-D is about 10 times as rapid as the breakdown revealed by assay of evolved  $^{14}\text{CO}_2$ , 3 as against  $0.3 \mu\text{mg/g}$  fresh wt./hr; and in the intact plant the breakdown as revealed by  $^{14}\text{CO}_2$  evolution from the roots (of the order of 3 of these units and the same as that given by bioassay of the cut tissue) is about one-tenth of that necessary to explain the vanishing of the applied dose. From these two discrepancies we may argue first that the cut tissue provides a less efficient system for 2,4-D breakdown as revealed by either assay. Whether this be from damage to the breakdown system on cutting or from a less efficient supply of 2,4-D to the site of breakdown in vacuum injection is a matter for further experiment to decide. Secondly, there is probably another fate that 2,4-D can suffer, which is not revealed by either assay, which accounts for the disappearance of a principal part of the free 2,4-D, and which is probably about 10 times as rapid in the intact plant as in cut tissue. On the basis of the evidence presented in Table 4, it is suggested that this process is the binding of the 2,4-D to proteins in the roots, followed by extensive metabolic breakdown. The binding occurs during the first 4 or 5 days, and at this time the 2,4-D is recoverable, but after this breakdown of the bound 2,4-D is rapid, and the amount recoverable falls rapidly. This mechanism seems to be the principal one by which it vanishes in this susceptible plant. The analogous behaviour of 2,4-D in resistant species is under investigation.

## V. ACKNOWLEDGMENTS

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## BREAKDOWN OF 2,4-D



Plant (Fig. 1) and radioautograph (Fig. 2) showing the rapid movement of labelled sugar from an application leaf to the root tips in 6 hr in contrast to the movement of 2,4-D shown in Figures 3 and 4. 2,4-D moves only to the upper part of the root system in 24 hr.

## BREAKDOWN OF 2,4-D



Dried plant (Fig. 1) and radioautograph (Fig. 2) of the Tick bean after respiration measurements which yielded the data of Table 2. Nearly all the  $^{14}\text{C}$  has moved from the shoot into the root in 4 days, but not beyond the upper part of the root system. 2,4-D has been converted there into many compounds (see text).

# ACCOMMODATION OF LINKAGE IN MASS SELECTION THEORY

By B. GRIFFING\*

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## *Summary*

The purpose of this paper is to develop mass selection theory which will accommodate not only linkage but will provide for different recombination frequencies in the two sexes.

The theoretical aspects of the linkage problem are developed in three stages:

- (1) The mass selection theory for two loci is extended to accommodate different recombination values for the two sexes.
- (2) A method is developed by which the generalized two-locus model may be used to cope with genetic situations which are considerably more complex. This method requires the estimation of the recombination value averaged over all possible pairs of loci.
- (3) The expectations of the half-sib and full-sib covariances for a random-mating population are generalized to permit different recombination values for the two sexes. This allows unbiased estimates of genotypic variance components to be obtained.

Finally, application of the more general mass selection theory to the problem of detecting the influence of natural selection in modifying the effectiveness of artificial selection, is discussed.

## I. INTRODUCTION

This paper is the second of a series in which the main objective is to generalize mass selection theory to include epistasis and linkage. Except for an abbreviated excursion by Kimura (1958), there has been no attempt to extend the mathematical theory of selection to include these phenomena. That is, no one has seriously attempted to explore selection theory using Kempthorne's (1954) generalized gene model which permits an exact treatment of epistasis. Likewise, no one has seriously attempted to solve the complex problem of linkage. Therefore, the past treatment of selection theory is inadequate, since, obviously, both epistasis and linkage are very real phenomena which should not be ignored.

In the first paper of this series (Griffing 1960), a hierarchical classification of hereditary units was considered. These units were the (1) gene, (2) gamete, and (3) individual. By using successively higher levels of hereditary units, successively higher levels of generalization of the selection theory were obtained. For example, when the individual was used as a unit of inheritance, the problem of linkage was avoided and certain very general statements were possible. However, by far the most informative approach was that in which the gamete was considered as the basic unit of inheritance, and the interpretation of the analysis was based on the gene.

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This gamete-gene approach is applied in the present paper. However, with this approach the linkage problem cannot be avoided and, in fact, since many experimental selection studies are conducted with *Drosophila*, it is necessary to handle not only the general concept of linkage, but it is also necessary to allow for the existence of different recombination values in the two sexes.

One approach to the linkage problem is to attempt to completely describe the genetic complexities (including all linkage parameters) in successively more complicated genetic systems (i.e. systems involving 2, 3, 4, . . . ,  $n$  loci). However, the algebra quickly becomes intractable.

In this study the linkage problem is attacked, first, by deriving the theoretical consequences of selection when a two-locus model is used which is generalized to accommodate different recombination values for the two sexes. This two-locus model is then adapted to approximately describe a complex situation by simply replacing the specific recombination value for the two loci by the recombination value *averaged over all possible pairs of loci*. It turns out that a sufficiently accurate estimate of this average recombination value can be obtained by a simple expression which is a function of only the recombination index. Finally, the problem of estimating certain variance components from covariances is solved by extending the covariance formulae to accommodate different recombination values in the two sexes.

It is immediately obvious from the general extension of the mass selection theory that certain epistatic effects cause results which mimic those due to the effects of natural selection. Therefore, a method is outlined with which it is possible to detect the influence of natural selection in modifying the responses to artificial selection even when epistasis is present.

## II. CONSEQUENCES OF TRUNCATION SELECTION FOR THE COMPLETELY GENERALIZED TWO-LOCUS MODEL

In the first paper of the series (Griffing 1960), the consequences of truncation selection based on the individual phenotype were examined in detail for the two-locus model which was completely general except for the fact that the recombination value was assumed to be the same for the two sexes. With regard to this assumption, the following conclusions were drawn:

"This simplification often does not exist. For example, an extreme case occurs in *Drosophila* where crossing over does not occur in the male. Thus, a somewhat more complicated analysis is required to accommodate different recombination values in the two sexes. However, such a complication does not change the general picture; it merely alters the speed of the response to selection and response to relaxation following selection."

In this section, then, the objective is to set out the analyses for the two-linked-locus case in which an arbitrary recombination frequency exists for each sex.

### (a) Definitions

It is assumed that the selection programme commences with a random-mating population which is in equilibrium. This population is designated as  $\Pi_0$ . The following notation is used (Kempthorne 1957):

Let

$$\sum_i p_i^1(A_i^1) = \text{array of alleles at locus (1),}$$

$$\sum_{ij} p_i^1 p_j^1(A_i^1 A_j^1) = \text{genotypic array at locus (1),}$$

$$\sum_k p_k^2(A_k^2) = \text{array of alleles at locus (2),}$$

$$\sum_{kl} p_k^2 p_l^2(A_k^2 A_l^2) = \text{genotypic array at locus (2),}$$

$y_f$  = recombination frequency between the two loci as exhibited by the females,

and

$y_m$  = recombination frequency between the two loci as exhibited by the males.

The initial equilibrium population may be generated as the product of the two genotypic arrays, i.e.

$$\begin{aligned}\Pi_0 &= [\sum_{ij} p_i^1 p_j^1(A_i^1 A_j^1)] [\sum_{kl} p_k^2 p_l^2(A_k^2 A_l^2)], \\ &= \sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2(A_i^1 A_j^1 A_k^2 A_l^2).\end{aligned}$$

Consider, now, the gametic arrays for each of the two sexes in  $\Pi_0$ . The female gametic array will be obtained first.

A female of the genotype  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  produces the following gametic array:

$$\{(1-y_f)/2\}(A_i^1 A_k^2 + A_j^1 A_l^2) + (y_f/2)(A_i^1 A_l^2 + A_j^1 A_k^2)\}.$$

Hence, the total gametic array for the females is

$$\sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 \{(1-y_f)/2\}(A_i^1 A_k^2 + A_j^1 A_l^2) + (y_f/2)(A_i^1 A_l^2 + A_j^1 A_k^2)\}.$$

This may be recast, using a summation device introduced by Kempthorne (1957), as follows:

$$\begin{aligned}\sum_{ijkl} \{p_i^1 p_j^1 p_k^2 p_l^2 [(1-y_f)/2] + p_j^1 p_i^1 p_l^2 p_k^2 [(1-y_f)/2] + p_i^1 p_j^1 p_l^2 p_k^2 (y_f/2) + p_j^1 p_i^1 p_k^2 p_l^2 (y_f/2)\} (A_i^1 A_k^2) \\ = \sum_{ik} ({}_f f_{ik}^0) (A_i^1 A_k^2),\end{aligned}$$

where

$$\begin{aligned}{}_f f_{ik}^0 &= \text{the relative frequency of the gamete } (A_i^1 A_k^2) \text{ produced by the females} \\ &\quad \text{in } \Pi_0 \\ &= p_i^1 p_k^2 \sum_{jl} \{(1-y_f)/2\}(2p_j^1 p_l^2) + (y_f/2)(2p_j^1 p_l^2) \\ &= p_i^1 p_k^2.\end{aligned}$$

Likewise, the frequency of the gamete  $(A_j^1 A_l^2)$  produced by the males in  $\Pi_0$  is

$${}_m f_{jl}^0 = p_j^1 p_l^2.$$

Thus, when the population is in equilibrium the frequency of a particular gamete produced by either males or females is simply the product of the appropriate component gene frequencies.

It is now possible to give another representation of the random-mating population in equilibrium in terms of the gamete as a unit of inheritance. This representation is that obtained by multiplying the gametic arrays from the two sexes as follows:

$$\begin{aligned} [\sum_{ik} (f f_{ik}^0)(A_i^1 A_k^2)] [\sum_{jl} (m f_{jl}^0)(A_j^1 A_l^2)] &= \sum_{ijkl} (f f_{ik}^0)(m f_{jl}^0)(A_i^1 A_k^2)(A_j^1 A_l^2) \\ &= \sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 (A_i^1 A_j^1 A_k^2 A_l^2), \end{aligned}$$

as before.

The genotypic value of  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  in  $\Pi_0$  is denoted as  $d_{ik,jl}$ , such that

$$\sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 d_{ik,jl} = 0.$$

This genotypic value is characterized by the following model (Kempthorne 1957):

$$\begin{aligned} d_{ik,jl} &= \alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2 + \delta_{ij}^1 + \delta_{kl}^2 + (\alpha\alpha)_{ik} + (\alpha\alpha)_{il} + (\alpha\alpha)_{jk} + (\alpha\alpha)_{jl} \\ &\quad + (\alpha\delta)_{ikl} + (\alpha\delta)_{jkl} + (\delta\alpha)_{ijk} + (\delta\alpha)_{ijl} + (\delta\delta)_{ijkl}, \end{aligned}$$

where

$\alpha_u^a$  = additive genetic effect of the  $A_u^a$  allele,

$\delta_{uv}^a$  = dominance effect associated with the  $A_u^a A_v^a$  genotype,

$(\alpha\alpha)_{ik}$  = additive  $\times$  additive epistatic effect associated with genes  $A_i^1$  and  $A_k^2$ ,

$(\alpha\delta)_{ikl}$  = additive  $\times$  dominance epistatic effect associated with the gene  $A_i^1$  and the genotype  $A_k^2 A_l^2$ , and

$(\delta\delta)_{ijkl}$  = dominance  $\times$  dominance epistatic effect associated with the genotypes  $A_i^1 A_j^1$  and  $A_k^2 A_l^2$ .

The total genotypic variance may be partitioned as

$$\sigma_G^2 = \sigma_A^2 + \sigma_D^2 + \sigma_{AA}^2 + \sigma_{AD}^2 + \sigma_{DD}^2,$$

where

$\sigma_G^2$  = total genotypic variance generated by the two loci,

$\sigma_A^2$  = additive genetic variance,

$\sigma_D^2$  = dominance variance,

$\sigma_{AA}^2$  = additive  $\times$  additive variance,

$\sigma_{AD}^2$  = additive  $\times$  dominance variance,

and

$\sigma_{DD}^2$  = dominance  $\times$  dominance variance.

In defining the selection value  $w_{ik.jl}$  for the genotype  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  it is assumed that the genotypic variability of the characteristic which is being studied is controlled by genes, each of small effect, at many loci, and that the phenotypic variability is normally distributed with mean zero and variance  $\sigma^2$ . Following Kimura (1958), the selection value  $w_{ik.jl}$  is defined to be proportional to the probability that an individual of the genotype  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  survives selection. Hence

$$w_{ik.jl} = 1 + (i/\sigma^2)d_{ik.jl},$$

where  $i$  is the selection differential. Details of the argument are presented in the earlier paper (Griffing 1960).

(b) *Consequences of n Generations of Continuous Selection*

The objective in this section is to describe the change in parameters which occurs with an arbitrary number of continuous cycles of selection. The procedure will be to outline briefly the method of obtaining the population mean which results from one generation of selection and then to consider the consequences of  $n$  consecutive cycles of selection.

The selection programme starts with a random-mating population in equilibrium, as described in the previous section. This population is designated as  $\Pi_0$ , and the populations resulting from successive cycles of selection are designated as  $\Pi_i$  ( $i = 1, \dots, n$ ).

The first cycle starts with

$$\Pi_0 = \sum_{ijkl} (f f_{ik}^0)(m f_{jl}^0)(A_i^1 A_k^2)(A_j^1 A_l^2).$$

The frequency (male or female) of the genotype  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  following selection is

$$(f f_{ik}^0)(m f_{jl}^0)w_{ik.jl}.$$

The total frequency of the selected individuals (male or female) is

$$\sum_{ijkl} (f f_{ik}^0)(m f_{jl}^0)[1 + (i/\sigma^2)d_{ik.jl}] = 1.$$

The first step in obtaining the mean of  $\Pi_1$  is to determine the frequency of a given gamete for each sex in the selected population. These give rise to gametic arrays for the selected males and females. The progeny mean,  $\mu_1$ , is then obtained by multiplying these gametic arrays and substituting the genotypic value for the genotypes. In the following, the frequency of the female gamete  $(A_i^1 A_k^2)$  will be obtained first. The objective is to determine the gametic frequency as a function of the parameters of  $\Pi_0$ .

The female genotype  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  produces the following gametic array:

$$\{[(1 - y_f)/2](A_i^1 A_k^2 + A_j^1 A_l^2) + (y_f/2)(A_i^1 A_l^2 + A_j^1 A_k^2)\}.$$



The total gametic array for the selected females is then,

$$\sum_{ijkl} (f f_{ik}^0)(m f_{jl}^0) w_{ik,jl}^0 \{[(1-y_f)/2](A_i^1 A_k^2 + A_j^1 A_l^2) + (y_f/2)(A_i^1 A_l^2 + A_j^1 A_k^2)\} = \sum_{ik} (f f_{ik}^1)(A_i^1 A_k^2),$$

where

$$\begin{aligned} f f_{ik}^1 &\cong [(1-y_f)/2][\sum_{jl} (f f_{ik}^0)(m f_{jl}^0) w_{ik,jl}^0 + (m f_{ik}^0) \sum_{jl} (f f_{jl}^0) w_{ik,jl}^0] \\ &\quad + (y_f/2)[\sum_{jl} (f f_{il}^0)(m f_{jk}^0) w_{il,jk}^0 + \sum_{jl} (f f_{jk}^0)(m f_{il}^0) w_{jk,il}^0] \\ &= [(1-y_f)/2][(f f_{ik}^0) + (m f_{ik}^0)] + (i/\sigma^2) p_i^1 p_k^2 [\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] + y_f p_i^1 p_k^2 \\ &= p_i^1 p_k^2 + (i/\sigma^2) p_i^1 p_k^2 [\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}]. \end{aligned}$$

Likewise, the frequency of the gamete  $(A_j^1 A_l^2)$  produced by the males is

$$\begin{aligned} m f_{jl}^1 &= [(1-y_m)/2][(f f_{jl}^0) + (m f_{jl}^0)] + (i/\sigma^2) p_j^1 p_l^2 [\alpha_j^1 + \alpha_l^2 + (\alpha\alpha)_{jl}] + (y_m) p_j^1 p_l^2 \\ &= p_j^1 p_l^2 + (i/\sigma^2) p_j^1 p_l^2 [\alpha_j^1 + \alpha_l^2 + (\alpha\alpha)_{jl}]. \end{aligned}$$

The structure of the population  $\Pi_1$  may now be written as

$$\Pi_1 = \sum_{ijkl} (f f_{ik}^1)(m f_{jl}^1)(A_i^1 A_k^2)(A_j^1 A_l^2),$$

which has the mean

$$\mu_1 = \sum_{ijkl} (f f_{ik}^1)(m f_{jl}^1) d_{ik,jl}^0.$$

This mean is approximately equal to

$$\sum_{ijkl} \{p_i^1 p_j^1 p_k^2 p_l^2 + (i/\sigma) p_i^1 p_j^1 p_k^2 p_l^2 [\alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2 + (\alpha\alpha)_{ik} + (\alpha\alpha)_{jl}]\} d_{ik,jl}^0 = (i/\sigma) [\sigma_A^2 + \frac{1}{2} \sigma_{AA}^2].$$

The approximation results from, first, making the transformation  $i = i/\sigma$  and, then, assuming that the term

$$\left( \frac{\text{gene effect}}{\text{total phenotypic standard deviation}} \right)$$

is small, so that the square or product of two such quantities can be neglected. Assumptions of this sort are made throughout this analysis.

The consequences of  $n$  generations of continuous selection may be outlined briefly as follows:

The population resulting from  $(n-1)$  consecutive cycles of selection has the following structure

$$\Pi_{n-1} = \sum_{ijkl} (f f_{ik}^{n-1})(m f_{jl}^{n-1})(A_i^1 A_k^2)(A_j^1 A_l^2).$$

The gametic array produced by the selected females is then

$$\sum_{ik} (f f_{ik}^n) (A_i^1 A_k^2),$$

where

$$\begin{aligned} f f_{ik}^n &\cong [(1-y_f)/2] (f f_{ik}^{n-1}) \sum_{jl} (m f_{jl}^{n-1}) w_{ik.jl}^0 + (m f_{ik}^{n-1}) \sum_{jl} (f f_{jl}^{n-1}) w_{ik.jl}^0 \\ &\quad + (y_f/2) [\sum_{jl} (f f_{jk}^{n-1}) (m f_{jk}^{n-1}) w_{ik.jl}^0 + \sum_{jl} (f f_{jk}^{n-1}) (m f_{il}^{n-1}) w_{jk.il}^0] \\ &= [(1-y_f)/2] [(f f_{ik}^{n-1}) + (m f_{ik}^{n-1})] + (i/\sigma^2) p_i^1 p_k^2 [\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] \\ &\quad + (y_f) p_i^1 p_k^2 + (n-1)(y_f)(i/\sigma^2) p_i^1 p_k^2 (\alpha_i^1 + \alpha_k^2). \end{aligned}$$

On converting to parameters of  $\Pi_0$  only, it appears that the above frequency can be put in the form

$$\begin{aligned} f f_{ik}^n &= [(1-y_f) b^{n-1} + y_f + a(1-y_f) \sum_{r=1}^{n-1} b^{r-1}] p_i^1 p_k^2 \\ &\quad + [1 + (1-y_f) \sum_{r=1}^{n-1} b^{r-1}] (i/\sigma^2) p_i^1 p_k^2 [\alpha_i^1 + \alpha_k^2 + (\alpha\alpha)_{ik}] \\ &\quad + [(n-1)(y_f) + \sum_{t=1}^{n-1} (\sum_{r=1}^t a(1-y_f) b^{r-1})] (i/\sigma^2) p_i^1 p_k^2 (\alpha_i^1 + \alpha_k^2) \\ &= p_i^1 p_k^2 + n(i/\sigma^2) p_i^1 p_k^2 (\alpha_i^1 + \alpha_k^2) + \{1 + (1-y_f)[(1-b^{n-1})/(1-b)]\} (i/\sigma^2) p_i^1 p_k^2 (\alpha\alpha)_{ik}, \end{aligned}$$

where

$$a = \frac{1}{2}(y_f + y_m),$$

and

$$b = 1 - a.$$

In a similar manner the frequency of the gamete  $(A_j^1 A_i^2)$  produced by the selected males is

$$m f_{ji} = p_j^1 p_i^2 + n(i/\sigma^2) p_j^1 p_i^2 (\alpha_j^1 + \alpha_i^2) + \{1 + (1-y_m)[(1-b^{n-1})/(1-b)]\} (i/\sigma^2) p_j^1 p_i^2 (\alpha\alpha)_{ji}.$$

Therefore, the mean of the population having  $n$  consecutive generations of selection can be determined as follows:

$$\begin{aligned} \mu_n &\cong \sum_{ijkl} (f f_{ik}^n) (m f_{jl}^n) d_{ik.jl}^0 \\ &= (i/\sigma^2) n \sigma_A^2 + [(1-b^n)/(1-b)] (i/\sigma^2) \frac{1}{2} \sigma_{AA}^2 \\ &= (i/\sigma^2) n \sigma_A^2 + (\sum_{r=1}^n b^{r-1}) (i/\sigma^2) \frac{1}{2} \sigma_{AA}^2 \\ &= (i/\sigma^2) (\sigma_A^2 + \frac{1}{2} \sigma_{AA}^2) + (i/\sigma^2) [\sigma_A^2 + (b) \frac{1}{2} \sigma_{AA}^2] \\ &\quad + (i/\sigma^2) [\sigma_A^2 + (b^2) \frac{1}{2} \sigma_{AA}^2] + \dots + (i/\sigma^2) [\sigma_A^2 + (b^{n-1}) \sigma_{AA}^2]. \end{aligned}$$

This is a more general expression for  $\mu_n$  than that previously obtained (Griffing 1960), when it was assumed that  $y_f = y_m = y$ . In this case,  $b = (1-y)$ .

(c) *Consequences of Relaxation after n Generations of Continuous Selection*

The objective in this section is to develop the prediction equation for the mean of a population which has had a history of  $n$  consecutive cycles of selection followed by  $t$  generations of random mating without selection. The procedure will be to start with  $\Pi_n$  and consider the consequences of one generation of relaxation, then to briefly outline the consequences of an arbitrary number  $t$  of generations of random mating without selection.

The notation is necessarily more complicated; thus  $(f_{ik}^{n,m})$  represents the frequency of the gamete  $(A_i^1 A_k^2)$  produced by the females selected from the population  $\Pi_{n,m-1}$  which has been subjected to  $n$  generations of continuous selection followed by  $(m-1)$  generations of random mating without selection.

To obtain the mean of  $\Pi_{n,1}$  it is necessary to start with

$$\Pi_{n,0} = \sum_{ijkl} (f_{ik}^{n,0})(m_{jl}^{n,0})(A_i^1 A_k^2)(A_j^1 A_l^2).$$

The female genotype  $(A_i^1 A_k^2)(A_j^1 A_l^2)$  produces the following gametic array

$$\{(1-y_f)/2\}(A_i^1 A_k^2 + A_j^1 A_l^2) + (y_f/2)(A_i^1 A_l^2 + A_j^1 A_k^2)\}.$$

The total gametic array for the female population in which there is no selection is

$$\sum_{ijkl} (f_{ik}^{n,0})(m_{jl}^{n,0})\{(1-y_f)/2\}(A_i^1 A_k^2 + A_j^1 A_l^2) + (y_f/2)(A_i^1 A_l^2 + A_j^1 A_k^2)\} = \sum_{ik} (f_{ik}^{n,1})(A_i^1 A_k^2),$$

where it can be shown that

$$f_{ik}^{n,1} = p_i^1 p_k^2 + n(i/\sigma^2)p_i^1 p_k^2(\alpha_i^1 + \alpha_k^2) + (i/\sigma^2)(1-y_f)\{(1-b^n)/(1-b)\}p_i^1 p_k^2(\alpha\alpha)_{ik}.$$

Likewise, the male frequency for the gamete  $(A_j^1 A_l^2)$  is

$$m_{jl}^{n,1} = p_j^1 p_l^2 + n(i/\sigma^2)p_j^1 p_l^2(\alpha_j^1 + \alpha_l^2) + (i/\sigma^2)(1-y_m)\{(1-b^n)/(1-b)\}p_j^1 p_l^2(\alpha\alpha)_{jl}.$$

Therefore, the mean of  $\Pi_{n,1}$  is

$$\begin{aligned} \mu_{n,1} &\cong \sum_{ijkl} (f_{ik}^{n,1})(m_{jl}^{n,1})a_{ik,jl}^0 \\ &= (i/\sigma^2)[\sigma_A^2 + (b)\frac{1}{2}\sigma_{AA}^2] + (i/\sigma^2)[\sigma_A^2 + (b)(b)\frac{1}{2}\sigma_{AA}^2] \\ &\quad + (i/\sigma^2)[\sigma_A^2 + (b)(b^2)\frac{1}{2}\sigma_{AA}^2] + \dots + (i/\sigma^2)[\sigma_A^2 + (b)(b^{n-1})\frac{1}{2}\sigma_{AA}^2]. \end{aligned}$$

By working through successive cycles, it is clear that after  $t$  generations of random mating without selection, the female and male gametic frequencies are:

$$f_{ik}^{n,t} = p_i^1 p_k^2 + n(i/\sigma^2)p_i^1 p_k^2(\alpha_i^1 + \alpha_k^2) + (i/\sigma^2)(1-y_f)\{(b^{t-1})[(1-b^n)/(1-b)]\}p_i^1 p_k^2(\alpha\alpha)_{ik},$$

and

$$m_{jl}^{n,t} = p_j^1 p_l^2 + n(i/\sigma^2)p_j^1 p_l^2(\alpha_j^1 + \alpha_l^2) + (i/\sigma^2)(1-y_m)\{(b^{t-1})[(1-b^n)/(1-b)]\}p_j^1 p_l^2(\alpha\alpha)_{jl}.$$

Hence, the mean of the population which has been subjected to  $n$  cycles of continuous selection followed by  $t$  generations of random mating without selection is

$$\begin{aligned}\mu_{n,t} &\cong \sum_{ijkl} (f f_{ik}^{n,t}) (m f_{jl}^{n,t}) d_{ik.jl}^0 \\ &= (i/\sigma^2) n \sigma_A^2 + (i/\sigma^2) \{ (b^t) [(1-b^n)/(1-b)] \} \frac{1}{2} \sigma_{AA}^2 \\ &= (i/\sigma^2) n \sigma_A^2 + (i/\sigma^2) [ (b^t) (\sum_{r=1}^n b^{r-1}) ] \frac{1}{2} \sigma_{AA}^2 \\ &= (i/\sigma^2) [\sigma_A^2 + (b^t) \frac{1}{2} \sigma_{AA}^2] + (i/\sigma^2) [\sigma_A^2 + (b^t)(b) \frac{1}{2} \sigma_{AA}^2] \\ &\quad + \dots + (i/\sigma^2) [\sigma_A^2 + (b^t)(b^{n-1}) \frac{1}{2} \sigma_{AA}^2],\end{aligned}$$

where, as before,

$$b = \{ [(1-y_f)/2] + [(1-y_m)/2] \} = [1 - (y_f + y_m)/2].$$

Again, this is a generalization of the previous result when it was assumed that  $y_f = y_m = y$ .

If the increment change in means for the  $(t-1)$ th and  $t$ th populations is defined as

$${}_n\Delta\mu_{(t-1),t} = \mu_{n,(t-1)} - \mu_{n,t},$$

then

$$\begin{aligned}{}_n\Delta\mu_{(t-1),t} &= (i/\sigma^2) \{ b^{t-1} [(1-b^n)/(1-b)] - b^t [(1-b^n)/(1-b)] \} \frac{1}{2} \sigma_{AA}^2 \\ &= (i/\sigma^2) [b^{t-1} (1-b^n)] \frac{1}{2} \sigma_{AA}^2.\end{aligned}$$

For any value of  $n$

$$\lim_{t \rightarrow \infty} [{}_n\Delta\mu_{(t-1),t}] \rightarrow 0,$$

hence the mean of the population  $\Pi_{n,0}$  decays to

$$\lim_{t \rightarrow \infty} (\mu_{n,t}) \rightarrow (i/\sigma^2) n \sigma_A^2.$$

Finally, it must be noted that in all of the analyses of this and the previous section, it is assumed that natural selection is not operating in any way to modify the pressure applied by artificial selection.

### III. ADAPTING THE TWO-LOCUS MODEL TO GENETICALLY MORE COMPLEX SITUATIONS

In the previous section the consequences of selection and relaxation from selection have been treated in detail for a very general genetic situation involving two loci. Clearly, however, if this form of analysis is to be of interest in selection theory, a method must be devised to adapt the two-locus model to accommodate, approximately, the variability generated by a much more complex genetic situation. This can only be done with certain simplifying assumptions. Thus, for the total genotypic variance, it is assumed that all epistatic interactions involving three



or more loci are negligible. For covariances which are disturbed by linkage, and for equations derived in the selection theory, it is assumed that: (1) all epistatic interactions involving three or more loci are negligible, and (2) the value for the covariance can be approximated by replacing the recombination value for the specific two-locus model by the recombination value averaged over all pairs of loci. The critical argument in this procedure is the argument on which the estimation of the "average recombination value" is based.

(a) *Average Recombination Value*

The problem in this section is to obtain an estimate of the recombination value averaged over all possible pairs of those loci whose genes cause variability in the characteristic under consideration.

The problem is particularly simple if all active loci are independent of each other in the segregational sense. In this case the average recombination value is, obviously,  $\frac{1}{2}$ . However, this situation implies that the individual loci are on different chromosomes, or, if two or more loci are on the same chromosome, they are spaced sufficiently far apart so that they segregate independently. Such restrictions make this simplified model unrealistic for most polygenic systems.

It is assumed in the following argument that for a given complexly inherited phenomenon, the number of active loci greatly exceeds the number of chromosome pairs, and that the genes are scattered over the chromosome complement. Hence, an entire range of linkage values among different pairs of loci is expected to occur. In this case it is obvious that the average recombination value over all pairs of loci lies between some lower limit and  $\frac{1}{2}$ .

In the first part of the following discussion, it will be shown that a lower limit can be determined quite simply using the chromosome as a unit of segregation. This argument will be followed by an attempt to bracket the average recombination value more exactly by taking crossing over into consideration. Finally it will be argued that a simple formula based only on the recombination index, although slightly biased, yields a sufficiently accurate estimate of the average recombination value for all practical purposes.

(b) *The Chromosome Argument*

For simplicity, consider the argument, first, for the situation of only two pairs of chromosomes. Assume that there are  $n_1$  loci on one pair and  $n_2$  loci on the other pair, where  $n_1 + n_2 = N$ . The total number of different pairwise combinations of loci is

$$\binom{N}{2} = \frac{N(N-1)}{2}.$$

Each of these combinations falls into one of three classes. These classes are:

- (i) Combinations in which both loci are located on the first chromosome.

The number of such pairs is

$$\binom{n_1}{2} = \frac{n_1(n_1-1)}{2}.$$

- (ii) Combinations in which both loci are located on the second chromosome. The number of such pairs is

$$\binom{n_2}{2} = \frac{n_2(n_2-1)}{2}.$$

- (iii) Combinations of loci, one of which is located on the first chromosome and the other on the second chromosome. There are  $n_1n_2$  such combinations.

Each pair of loci in classes (i) and (ii) may exhibit a recombination value between 0 and  $\frac{1}{2}$ . However, it is assumed that each and every pair of loci in class (iii) exhibits a recombination value of exactly  $\frac{1}{2}$ . Hence, the lowest possible limit for the recombination value averaged over all possible pairs of loci is obtained by setting all recombination values in classes (i) and (ii) equal to zero. This lowest value is then

$$\frac{1}{2}[(n_1n_2)/\frac{1}{2}N(N-1)] = n_1n_2/N(N-1).$$

If  $n_1 \cong N/2$ , then the lowest average value becomes

$$N^2/4(N^2-N) = 1/4[1-(1/N)],$$

and the limit of this value as  $N$  becomes large is  $\frac{1}{4}$ .

This argument can be generalized easily to any number,  $m$ , of non-homologous chromosome sets. Suppose that there are  $n_i$  loci on the  $i$ th chromosome set ( $i = 1, \dots, m$ ) such that  $N = \sum n_i$ . The total number of different pairs of loci is

$$\binom{N}{2} = \frac{N(N-1)}{2}.$$

The number of pairs of loci, one on each of two non-homologous chromosomes is

$$\sum_{i < j} n_i n_j, \quad (i, j = 1, \dots, m).$$

Hence, the lowest possible average recombination value is

$$\sum_{i < j} n_i n_j / N(N-1). \quad \dots \dots \dots (1)$$

If  $n_i \cong (1/m)N$  (for all  $i$ ), then (1) becomes

$$[(m-1)/2m][N^2/(N^2-N)] = [(m-1)/2m]\{1/[1-(1/N)]\}.$$

and the limit of this value as  $N$  becomes large is  $(m-1)/2m$ .

The above argument has been given in terms of chromosomes, and the lower limit of the average recombination value has been derived on the basis of no crossing over. In this case meiosis results in the independent segregation of entire chromosomes whose loci are completely linked. If crossing over occurs, it is possible to state that the recombination value averaged over all possible pairs of active loci lies in the interval

$$(m-1)/2m < \bar{y} < \frac{1}{2}.$$

It is instructive to tabulate  $(m-1)/2m$  for varying values of  $m$  as follows ( $m$  = number of chromosome pairs):

$m$	1	2	3	4	5	6	10	20
$(m-1)/2m$	0	1/4	1/3	3/8	2/5	5/12	9/20	19/40

An interesting fact immediately becomes clear from this table, viz. the recombination value averaged over all possible pairs of loci is close to  $\frac{1}{2}$  if the haploid chromosome number is five or more. This is due to the fact that as the number of chromosomes increases, the relative proportion of linked pairs of loci rapidly decreases and the average recombination value asymptotes steeply towards  $\frac{1}{2}$ .

However, there are undoubtedly many instances when attention is focused on sets of chromosomes whose numbers are small (i.e. less than five). This is particularly true for *Drosophila melanogaster*, since it is extensively used as an experimental organism in testing quantitative inheritance and selection theories. Therefore, it is of interest to extend the above argument in some detail.

#### (c) The Crossing Over Argument

The estimation of the average recombination value can be made more exact by considering the consequences of crossing over. This phenomenon increases the number of segregating units, and therefore, it is natural to suppose that an estimate of the average recombination value can be obtained by replacing  $m$  in the "chromosome" formula by Darlington's (1958) recombination index. This index gives the average number of pieces into which the chromosome complement is divided by chiasma formation.

However, this procedure, which may be termed the index method of estimation, is biased. This is so because the actual pattern of chiasma formation yields an extensive array of chromosome segments having different numbers of active loci, rather than a constant pattern of segments all having approximately the same number of loci. It will now be shown that, because of this fact, the index method yields an upper limit to the average recombination value. The argument will be illustrated first, for an obligatory chiasma forming on a single chromosome pair.

Suppose that a single chiasma forms at exactly the same position on the given chromosome pair in every mother cell. There will be  $n_1$  active loci to the left and  $n_2$  active loci to the right of the exchange. Clearly, pairs of loci to the left exhibit a recombination value of zero; as do pairs of loci to the right of the chiasma position. However, pairs of loci, which involve one locus on each side of the chiasma, exhibit a recombination value of  $\frac{1}{2}$ . Hence, the recombination value averaged over all loci is

$$\begin{aligned}\bar{y} &= \frac{1}{2}n_1n_2 / \binom{N}{2} \\ &= n_1n_2/N(N-1).\end{aligned}$$

It can be shown that the average recombination value,  $\bar{y}$ , varies depending on the position of the chiasma, which in turn alters the relative magnitudes of  $n_1$  and  $n_2$ . Furthermore, it can be shown that the maximum value of  $\bar{y}$  is obtained for the situation in which  $n_1 = n_2$ . The argument may be sketched as follows: Let

$$N = n_1 + n_2,$$

$$n_1 = n_2(1 + \delta),$$

where  $\delta > -1$ . Then

$$n_1 = N[(1 + \delta)/(2 + \delta)],$$

and

$$n_2 = N/(2 + \delta).$$

Hence

$$\begin{aligned}\bar{y} &= [(1 + \delta)/(2 + \delta)^2] \{1/[1 - (1/N)]\} \\ &\cong (1 + \delta)/(2 + \delta)^2, \quad \text{for large } N, \\ &= 1/\{4 + [\delta^2/(1 + \delta)]\}.\end{aligned}$$

Since  $\delta > -1$ ,  $\delta^2/(1 + \delta) \geq 0$ . Therefore, the maximum value of  $\bar{y}$  is  $\frac{1}{4}$ , which occurs when  $\delta = 0$ . This is the value obtained by the index estimation method.

This proof can be extended to the situation of more than one obligatory chiasma. The following argument for two chiasmata illustrates how this may be done.

Consider a hypothetical situation in which two chiasmata invariably form a given pattern in every mother cell. Let there be  $n_1$  active loci to the left of the first chiasma,  $n_2$  loci between the two chiasmata, and  $n_3$  loci to the right of the second chiasma. Assuming no chromatid interference, the average recombination value over all pairs of loci is then,

$$\begin{aligned}\bar{y} &= \frac{1}{2}(n_1n_2 + n_1n_3 + n_2n_3) / \binom{N}{2} \\ &= (n_1n_2 + n_1n_3 + n_2n_3)/N(N - 1).\end{aligned}$$

The maximum value for  $\bar{y}$  can be determined as follows:

Let

$$N = n_1 + n_2 + n_3,$$

$$n_1 = n_2(1 + \alpha),$$

where  $\alpha > -1$ , and

$$n_3 = n_2(1 + \beta),$$

where  $\beta > -1$ . Then

$$n_1 = [(1 + \alpha)/(3 + \alpha + \beta)]N, \quad n_2 = N/(3 + \alpha + \beta), \quad \text{and} \quad n_3 = [(1 + \beta)/(3 + \alpha + \beta)]N.$$

Hence

$$\bar{y} = \frac{3 + 2\alpha + 2\beta + \alpha\beta}{(3 + \alpha + \beta)^2} \left[ \frac{1}{1 - (1/N)} \right],$$

which, for large  $N$ , is approximately

$$1 / \left[ 3 + \frac{\alpha^2 + \beta^2 - \alpha\beta}{3 + 2\alpha + 2\beta + \alpha\beta} \right].$$



Since  $\alpha, \beta > -1$ ,

$$(\alpha^2 + \beta^2 - \alpha\beta)/(3 + 2\alpha + 2\beta + \alpha\beta) \geq 0.$$

Thus,  $\bar{y}$  is a maximum when

$$(\alpha^2 + \beta^2 - \alpha\beta)/(3 + 2\alpha + 2\beta + \alpha\beta) = 0,$$

and this occurs only when both  $\alpha$  and  $\beta$  equal zero. Therefore, the maximum value occurs when, invariably,  $n_1 = n_2 = n_3$ : the situation required for the argument involving the recombination index.

It is clear, then, that any agency which causes the positions of the chiasmata to be varied so that the chromosome pieces do not have equal contents of active loci lowers the average recombination value.

In reality, of course, the pattern of chiasmata is not invariable. Generally, chiasmata may form along the entire length of the chromosome, and the number of chiasmata for any given chromosome pair may vary in different mother cells. Observational data on the distribution of chiasmata in *individual* chromosomes are few, perhaps the most extensive are those reported by White and Morley (1955). However, it is apparently agreed that the following two conditions hold for most species of plants and animals:

- (1) At least one chiasma per bivalent is obligatory for the survival of the bivalent; and
- (2) A strong chiasma interference exists, at least within each arm of every chromosome.

Since there is no chiasma distributional theory which completely satisfies the above conditions, the procedure which will be followed is to continue the approach of using simplified cross-over models to bracket the true recombination value in as small an interval as possible. Finally, it will be shown that the index method, although biased, yields a sufficiently accurate estimate for most practical situations.

In view of the fact that the pattern of chiasmata is not invariable, it is clear that the index method yields an upper limit to the average recombination value. Therefore the true average recombination value must lie in the interval

$$(m-1)/2m < \bar{y} < (r-1)/2r,$$

where  $m$  = haploid chromosome number, and  $r$  = recombination index.

In this interval, the upper limit is set by a cross-over model in which the chiasma configuration invariably yields chromosomal segments containing equal numbers of active loci. It is "sensitive" in that the limit changes with different numbers of chiasmata for a given number of chromosomes. The lower limit, however, is based on chromosomal segregation and is "insensitive" to chiasma distribution. Hence the next step is to devise a cross-over model which yields a sensitive lower limit to the true average recombination value.

As mentioned earlier, one of the accepted facts with regard to actual chiasma distribution is the strong chiasma interference within a chromosome arm. Such interference tends to space the chiasmata located in the same arm. This results in a restricted array of chromosomal segments tending to have the same number

of active loci: the condition required by the index method. Hence, a cross-over model in which there is no chiasma interference will produce an unrestricted array of chromosome segments, with the net result that the average recombination value based on this model will generally be lower than the true value. Thus, a non-interference model, incorporating the following conditions, will be considered as providing a sensitive lower limit for the true average recombination value:

- (1) At least one obligatory chiasma per bivalent;
- (2) Non-interference of chiasma formation, i.e. all chiasma form independently of each other; and
- (3) No chromatid interference.

In determining the consequences of this non-interference model, it is convenient to break down the approach into two stages, both of which are concerned with chiasma formation on a *single chromosome pair*. In stage one, the average recombination value is determined for a given number,  $k$ , of independent chiasmata formed on the single chromosome pair in every mother cell. In stage two, the numbers of chiasmata are allowed to vary according to the Poisson distribution.

With regard to stage one, first consider a specific example in which there are three independent chiasmata formed on the given pair of chromosomes in every mother cell. Let the length of the chromosome be divided by  $n$  loci into  $(n-1)$  regions, in each of which a chiasma is equally likely to occur. (It is assumed that the regions are sufficiently small so that the probability of two chiasmata forming in the same region is negligible).

There are  $\binom{n}{2}$  different pairwise combinations of loci, and  $\binom{n-1}{3}$  different

chiasma configurations. Hence, there is a total of  $\binom{n}{2}\binom{n-1}{3}$  events, which, for a given pair of loci, may be defined in terms of the recombinational consequences of the imposition of a certain chiasma configuration. Thus a recombinant event is one which, when all possible meiotic configurations are considered, results in 50 per cent. recombinant chromosomes. Such an event occurs when at least one chiasma forms between the two loci, and a non-recombinant event occurs when chiasmata do not form between the loci.

The recombination value averaged over all possible pairs of loci may then be defined as

$$\bar{y} = \left( \frac{\text{Number of recombinant events}}{\text{Total number of events}} \right) \times \frac{1}{2}.$$

This is most easily obtained as

$$\bar{y} = \left( \frac{(\text{Total number of events}) - (\text{Number of non-recombinant events})}{\text{Total number of events}} \right) \times \frac{1}{2}.$$

The basic problem, then, is the enumeration of the non-recombinant events.

For a given chromosome configuration, a non-recombinant event exists when the region between the two loci is not interrupted by one or more chiasmata. Hence, for the three-chiasmata example, the enumeration problem is simplified if, for each chiasma configuration, the number of combinations of loci which occur, (1) to the left of the first chiasma, (2) between the first and second chiasmata, (3) between the second and third chiasmata, and (4) to the right of the third chiasma, are enumerated. The total number of such non-recombinants is found to be

$$4 \sum_{r=2}^{n-3} \binom{n-r-1}{2} \binom{r}{2}.$$

Therefore, the average recombination value, as  $n$  increases indefinitely, for a single chromosome pair invariably having three chiasmata is

$$\begin{aligned} \bar{y} &= \lim_{n \rightarrow \infty} \left[ \frac{\binom{n}{2} \binom{n-1}{3} - 4 \sum_{r=2}^{n-3} \binom{n-r-1}{2} \binom{r}{2}}{\binom{n}{2} \binom{n-1}{3}} \right] \times \frac{1}{2} \\ &= \lim_{n \rightarrow \infty} \left[ \frac{(1/12)n(n-1)^2(n-2)(n-3) - (1/30)n(n-1)(n-2)(n-3)(n-4)}{(1/12)n(n-1)^2(n-2)(n-3)} \right] \times \frac{1}{2} \\ &= \lim_{n \rightarrow \infty} \left\{ 3/10 \left[ \frac{1 + (1/n)}{1 - (1/n)} \right] \right\} \\ &= 3/10. \end{aligned}$$

This argument can be readily generalized to any number,  $k$ , of independent chiasmata formed on the given pair of homologous chromosomes in each mother cell. This generalization results in the following expressions for the average recombination value:

$$\bar{y} = \lim_{n \rightarrow \infty} \left\{ \frac{\binom{n}{2} \binom{n-1}{k} - (k+1) \sum_{r=2}^{n-k} \binom{n-r-1}{k-1} \binom{r}{2}}{\binom{n}{2} \binom{n-1}{k}} \right\} \times \frac{1}{2}$$

which is, apparently,

$$\begin{aligned} &= \lim_{n \rightarrow \infty} \left\{ \frac{\frac{1}{2k!} \left[ \frac{n(n-1)(n-1)!}{(n-k-1)!} \right] - \left[ \frac{n!}{k!(k+2)(n-k-2)!} \right]}{\frac{1}{2k!} \left[ \frac{n(n-1)(n-1)!}{(n-k-1)!} \right]} \right\} \times \frac{1}{2} \\ &= \lim_{n \rightarrow \infty} \left\{ \left( \frac{k}{k+2} \right) \left( \frac{1 + (1/n)}{1 - (1/n)} \right) \right\} \times \frac{1}{2} \\ &= k/2(k+2). \end{aligned}$$

The above argument is developed for the genetic situation in which exactly  $k$  independent chiasmata occur on the given bivalent in every mother cell. It is now necessary to extend this argument to permit varying numbers of chiasmata to form on the chromosome pair in different mother cells. The extension must satisfy the two conditions mentioned previously, i.e. (1) at least one chiasma is obligatory for each bivalent, and (2) all chiasmata form independently of each other (no chiasma interference). It follows from the last condition that, apart from the initial obligatory chiasma, the numbers of additional chiasmata are distributed according to the Poisson distribution. Thus, the probability of exactly  $t$  additional chiasmata is

$$P(t; \lambda) = \frac{e^{-\lambda} \lambda^t}{t!},$$

where  $\lambda = (\text{average number of chiasmata}) - 1$ .

The frequencies for varying  $t$  values are as follows:

$t$	0	1	2	3	...
Obligatory chiasma	1	1	1	1	...
Total chiasmata ( $k = t + 1$ )	1	2	3	4	...
Frequency	$e^{-\lambda}$	$e^{-\lambda} \lambda$	$(e^{-\lambda} \lambda^2)/2!$	$(e^{-\lambda} \lambda^3)/3!$	...

The average recombination value for *exactly*  $k$  chiasmata in each and every cell may be recast in terms of the variable  $t$  as follows:

$$\begin{aligned} \bar{y} &= \frac{k}{2(k+2)} \\ &= \frac{(t+1)}{2(t+3)}. \end{aligned}$$

The average recombination value over all possible pairs of loci may now be obtained for the situation in which the frequencies for varying values of  $t$  are taken into consideration as follows:

$$\begin{aligned} \bar{y} &= \sum_{t=0}^{\infty} [(e^{-\lambda} \lambda^t)/t!] [(t+1)/2(t+3)] \\ &= \sum_{t=0}^{\infty} [(e^{-\lambda} \lambda^t)/t!] [\tfrac{1}{2} - 1/(t+3)] \\ &= \tfrac{1}{2} \sum_{t=0}^{\infty} [(e^{-\lambda} \lambda^t)/t!] - (1/e^{\lambda}) \{ \sum_{t=0}^{\infty} [\lambda^t/t!(t+3)] \} \\ &= \tfrac{1}{2} - (1/e^{\lambda}) \{ (2/\lambda^3) [(\lambda^2 e^{\lambda}/2) - (\lambda - 1)e^{-\lambda} - 1] \} \\ &= \tfrac{1}{2} - (1/\lambda) + [2(\lambda - 1)/\lambda^3] + (2/\lambda^3 e^{\lambda}). \end{aligned}$$



The value  $\bar{y}$  then is the recombination value averaged over all possible pairs of loci for a single chromosome pair when it is assumed that:

- (1) a large number of active loci occur at random in the chromosome pair;
- (2) at least one chiasma is obligatory for the survival of the bivalent; and
- (3) there is neither chiasma nor chromatid interference.

The above expression holds for  $\lambda \geq 1$ ; for  $\lambda = 0$ ,  $\bar{y} = \frac{1}{6}$ .

To summarize the results when crossing over is taken into consideration, the true average recombination value for a single chromosome pair lies in the interval defined

TABLE 1  
DIFFERENCE OF THE AVERAGE RECOMBINATION VALUE FOR THE METHOD IN WHICH THE RECOMBINATION INDEX IS USED AND THE METHOD IN WHICH THE CHIASMATA ARE ASSUMED TO FORM INDEPENDENTLY OF EACH OTHER WITH THE RESTRICTION THAT AT LEAST ONE CHIASMA IS OBLIGATORY

Average No. of Chiasmata per Chromosome ( <i>s</i> )	No. of Sets of Homologous Chromosomes ( <i>m</i> )				
	1	2	3	4	5
1	0.083	0.042	0.028	0.021	0.017
2	0.098	0.049	0.033	0.024	0.020
3	0.091	0.046	0.030	0.023	0.018
4	0.081	0.041	0.027	0.020	0.016
5	0.072	0.036	0.024	0.018	0.014

by the non-interference model at the lower limit and the index model at the upper limit, i.e.

- (1) Single obligatory chiasma:

$$\frac{1}{6} < \bar{y} < \frac{1}{4}.$$

- (2) Single obligatory chiasma plus  $\lambda$  additional chiasmata:

$$\left\{ \frac{1}{2} - (1/\lambda) + [2(\lambda-1)/\lambda^3] + (2/\lambda^3 e^\lambda) \right\} < \bar{y} < [(\lambda+1)/2(\lambda+2)].$$

The magnitudes of the intervals for different average numbers of chiasmata are given in the first column of Table 1. These values vary from 7 to 10 per cent. and tend to diminish as the average number of chiasmata increases. Because (i) it is likely that the probability distribution of chiasma position is not uniform throughout a chromosome arm, and (ii) a powerful chiasma interference occurs which tends to disperse the points of exchange, the true average recombination value, in most instances, will probably lie toward the upper limit of the interval, not far from the value given by the index method. For a single chromosome, then, the index method yields an estimate with a slight positive bias.

In selection theory, however, one is seldom concerned with a single chromosome; therefore, it is of interest to determine the magnitude of the interval generated by the non-interference (independent chiasmata) and the index methods, as the chromosome number increases. The following gives the details for (i)  $m$  chromosome pairs each having only one chiasma, and (ii)  $m$  chromosome pairs each having an average of  $s = (\lambda + 1)$  chiasmata.

For the situation in which each of  $m$  chromosome pairs has only one chiasma, the two models yield the following formulae:

$$\hat{y}_{\text{index}} = (2m-1)/4m,$$

and

$$\hat{y}_{\text{indep.}} = (3m-2)/6m.$$

Hence, the difference between the two estimators is

$$d = \hat{y}_{\text{index}} - \hat{y}_{\text{indep.}} = 1/12m.$$

For the situation in which each of  $m$  chromosome pairs has an average of  $s = (\lambda + 1)$  chiasmata, the corresponding formulae are

$$\hat{y}_{\text{index}} = [m(\lambda + 2) - 1]/[2m(\lambda + 2)],$$

$$\hat{y}_{\text{indep.}} = \frac{1}{2} - (1/m\lambda) + [2(\lambda - 1)/m\lambda^3] + 2e^{-\lambda}/m\lambda^3,$$

and

$$d = \hat{y}_{\text{index}} - \hat{y}_{\text{indep.}} = \{(\lambda^3 - 4\lambda + 8)/[2m(\lambda + 2)\lambda^3]\} - (2e^{-\lambda}/m\lambda^3).$$

The magnitudes of the differences for varying values of  $m$  and  $s$  are given in Table 1. It is clear that as the number of chromosomes increases, the differences generated by the two methods of estimation diminish, and both methods converge on the true value.

It may be concluded that for all practical purposes the index method, which is simple and convenient to use, yields a satisfactory estimate of the average recombination value. For example, when the chromosome number is increased to only two, the true recombination value may be expected to lie within 2 per cent. of the value given by the index method. If greater precision is required, estimates could be made using both methods and the arithmetic mean obtained from them.

The methods outlined in this section give at least a first approximation to the recombination value averaged over all pairs of loci. The approximation becomes better as more information on chiasma frequency becomes available. If only the chromosome number is known, one can certainly say that the average recombination value lies in the range

$$(m-1)/2m < \hat{y} < \frac{1}{2},$$

where  $m$  is the haploid chromosome number. If fertility is high, it can be assumed that at least one obligatory chiasma occurs in each and every chromosome pair. Hence, without actually making a chiasma count, one can obtain an estimate of the

average recombination value, which should be fairly accurate in most cases, by the following formula

$$\bar{y} = (m' - 1)/2m',$$

where  $m'$  is twice the haploid chromosome number.

Finally, if the average number of chiasmata per cell is known, it is possible to bracket the average recombination value in a smaller range, i.e.

$$\left\{ \frac{1}{2} - \frac{1}{p-m} + \frac{2m^2}{(p-m)^3} \left[ \exp\left(-\frac{p-m}{m}\right) + \frac{p-2m}{m} \right] \right\} < \bar{y} < \frac{p+m-1}{2(p+m)},$$

where the expression to the left is the average recombination value for the non-interference model when there is an average of  $(\lambda+1) = p/m$  chiasmata on each of  $m$  chromosome pairs, and  $p$  is the average number of chiasmata per nucleus. When the number of chiasmata vary with different chromosomes, this expression is not exact. The expression on the right is the average recombination value given by the index method.

For practical purposes a direct estimate of the average recombination value may be made, using the convenient and simple index formula, i.e.

$$\bar{y} = (p+m-1)/2(p+m).$$

The above argument supposes that (i) there are a very large number of active loci scattered at random over the chromosome set, (ii) the chromosomes are not drastically different in size (as, for example, with the two major and the fourth autosomes in *Drosophila*), and (iii) a chiasma is invariably associated with a genetic crossing over.

#### (d) *A Drosophila Example*

Clayton, Morris, and Robertson (1957) and Clayton and Robertson (1957) demonstrated that with the population of *Drosophila* which they were using, almost all of the genetic variability for abdominal bristle number was generated by genes located in the two major autosomes. The question may now be asked as to what is the average recombination value for each of the two sexes for all possible pairs of genes causing variability in bristle number.

Since crossing over does not occur in the male, the recombination index for these autosomes is merely 2, and hence the average recombination value for males is equal to  $\frac{1}{4}$ .

Crossing over occurs in the female, enabling linkage maps to be synthesized. Since the map for each of the two major autosomes is slightly greater than 100 cross-over units, the recombination index for the two major autosomes is approximately 6. Therefore, the average recombination value for females is approximately 5/12.

#### IV. ESTIMATES OF VARIANCE COMPONENTS FROM COVARIANCES WHEN THE RECOMBINATION VALUES ARE DIFFERENT IN THE TWO SEXES

In a previous section formulae were given for the responses to selection and relaxation from selection in terms of genotypic variance components. In comparing

the theoretical consequences of selection with the experimental results, it is necessary to estimate these variance components from the original random-mating population. This is done with covariances.

The expectations of some of these covariances are affected by linkage, and Cockerham (1956) has given the expectations of the necessary covariances for the situation in which the recombination value is the same for both sexes. These expressions must now be generalized to accommodate different recombination values for the two sexes.

There are three covariances which are of interest. These are: parent-offspring covariance, designated as  $\text{Cov}(\text{PO})$ ; half-sib covariance, designated as  $\text{Cov}(\text{HS})$ ; and full-sib covariance, designated as  $\text{Cov}(\text{FS})$ . Of these, the  $\text{Cov}(\text{PO})$  is not affected by linkage, but linkage parameters do enter into the expectations for the other two covariances.

The definition of the half-sib covariance may be reduced to the expectation of the squares of the half-sib family means. A "sire" half-sib family is generated by the union of the gametic array of an arbitrary sire with the total gametic array from the dams, and, similarly, a "dam" half-sib family results from the union of the gametic array of an arbitrary dam with the total gametic array from the sires. If the recombination value is different for the two sexes, it is obvious that the sire and dam half-sib families differ, even if the sire and dam are of the same genotype. Thus, two different covariances are possible:

$\text{Cov}_{(m)}(\text{HS}) = \text{covariance generated by sire half-sib families, and}$

$\text{Cov}_{(f)}(\text{HS}) = \text{covariance generated by dam half-sib families.}$

Consider first, the derivation of  $\text{Cov}_{(m)}(\text{HS})$ . An arbitrary sire  $(A_i^1 A_k^2)(A_j^1 A_l^2)$ , produces the following gametic array

$$\{(1-y_m)/2\}(A_i^1 A_k^2 + A_j^1 A_l^2) + (y_m/2)(A_i^1 A_l^2 + A_j^1 A_k^2)\}.$$

The total female gametic array in the random-mating population is

$$\sum_{rt} p_r^1 p_t^2 (A_r^1 A_t^2).$$

Hence, the sire half-sib family mean is

$$\begin{aligned} h_{(ik,jl)}(\dots) &= [(1-y_m)/2][\sum_{rt} p_r^1 p_t^2 d_{tk,rt} + \sum_{rt} p_r^1 p_t^2 d_{jl,rt}] \\ &\quad + (y_m/2)[\sum_{rt} p_r^1 p_t^2 d_{il,rt} + \sum_{rt} p_r^1 p_t^2 d_{jk,rt}]. \end{aligned}$$

The sire half-sib covariance may then be evaluated as

$$\begin{aligned} \text{Cov}_{(m)}(\text{HS}) &= \sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 [h_{(ik,jl)}(\dots)]^2 \\ &= \frac{1}{4} \sigma_A^2 + [(1/16) + (\delta_m/16)] \sigma_{AA}^2, \end{aligned}$$

where

$$\delta_m = (1-2y_m)^2.$$



Likewise, the dam half-sib covariance is

$$\text{Cov}_{(f)}(\text{HS}) = \frac{1}{4}\sigma_A^2 + [(1/16) + (\delta_f/16)]\sigma_{AA}^2,$$

where

$$\delta_f = (1 - 2y_f)^2.$$

The definition of the full-sib covariance may be reduced to the expected value of the squares of the full-sib means. Consider, now, the evaluation of this definition for the case of different recombination values for the two sexes.

The mean of the full-sib array which results from the cross between an arbitrary sire,  $(A_i^1 A_k^2)(A_j^1 A_l^2)$ , and an arbitrary dam,  $(A_r^1 A_t^2)(A_s^1 A_u^2)$ , is

$$\begin{aligned} h_{(ik,jl)(rt,su)} = & \{[(1-y_m)/2][(1-y_f)/2](d_{ik,rt} + d_{ik,su} + d_{jl,rt} + d_{jl,su}) \\ & + [(1-y_m)/2](y_f/2)(d_{ik,ru} + d_{ik,st} + d_{jl,ru} + d_{jl,st}) \\ & + (y_m/2)[(1-y_f)/2](d_{il,rt} + d_{il,su} + d_{jk,rt} + d_{jk,su}) \\ & + (y_m/2)(y_f/2)(d_{il,ru} + d_{il,st} + d_{jk,ru} + d_{jk,st})\}. \end{aligned}$$

By definition, the full-sib covariance may be obtained as follows:

$$\begin{aligned} \text{Cov}(\text{FS}) = & \sum_{ijklrstu} p_i^1 p_j^1 p_r^1 p_s^1 p_k^2 p_l^2 p_t^2 p_u^2 [h_{(ik,jl)(rt,su)}]^2 \\ = & \frac{1}{2}\sigma_A^2 + \frac{1}{4}\sigma_D^2 + \left\{\frac{1}{4} + [(\delta_f + \delta_m)/16]\right\}\sigma_{AA}^2 \\ & + \left\{\frac{1}{8} + [(\delta_f + \delta_m)/16]\right\}\sigma_{AD}^2 + (1/16)(1 + \delta_f)(1 + \delta_m)\sigma_{DD}^2. \end{aligned}$$

Assuming that the epistatic interactions involving three or more loci are negligible, the simplest method of estimating  $\sigma_A^2$  and  $\sigma_{AA}^2$  is to use the parent-offspring and half-sib covariances. Thus, starting with the expectations

$$\text{Cov}(\text{PO}) = \frac{1}{2}\sigma_A^2 + \frac{1}{4}\sigma_{AA}^2,$$

and

$$\text{Cov}_{(m)}(\text{HS}) = \frac{1}{4}\sigma_A^2 + 1/16(1 + \delta_m)\sigma_{AA}^2,$$

estimates of  $\sigma_A^2$  and  $\sigma_{AA}^2$  may be obtained as follows:

$$\hat{\sigma}_A^2 = \{\text{Cov}(\text{PO})[2y_m(1-y_m)-1] + 2[\text{Cov}_{(m)}(\text{HS})]\}/y_m(1-y_m),$$

and

$$\hat{\sigma}_{AA}^2 = 2\{\text{Cov}(\text{PO}) - 2[\text{Cov}_{(m)}(\text{HS})]\}/y_m(1-y_m).$$

Normally, then, the experimental procedure would be to obtain accurate estimates of the parent-offspring and half-sib covariances from the original random-mating population, and compute  $\hat{\sigma}_A^2$  and  $\hat{\sigma}_{AA}^2$  using the above expressions in which the specific recombination values are replaced by the recombination value averaged over all possible pairs of active loci. The variance estimates may then be substituted into the theoretical selection formulae for comparison with the observed responses.

# V. A THEORETICAL METHOD FOR THE DETECTION OF DISTURBANCES DUE TO NATURAL SELECTION

The selection theory has been developed on the basis that *natural* selection is not operating differentially on the various genotypes. Clearly, however, this need not be the case. It appears that in experiments where artificial selection has been applied unidirectionally, it has often been found that when the population is relaxed from selection, the mean regresses toward its original value. This phenomenon has been termed "genetic homeostasis" by Lerner (1954), and it is generally assumed that this regression is due to the effects of natural selection which oppose the effects of artificial selection.

However, in this study, and in a previous paper (Griffing 1960), it is shown that the contributions of certain epistatic effects mimic the antagonistic effects of natural selection. This mimicry occurs in both the response to selection and relaxation from selection. Thus, even when natural selection is ineffective, the increment changes due to artificial selection in successive generations become increasingly smaller: i.e. in the formula

$$\Delta\mu_{n,(n-1)} = (i/\sigma^2)[\sigma_A^2 + (b^{n-1})\frac{1}{2}\sigma_{AA}^2],$$

the contribution of  $\sigma_{AA}^2$  decreases as  $n$  increases. This diminishing of increments is similar to the effect that one would expect on the assumption that the intensity of natural selection increases as artificial selection causes the cumulative change in the population mean to increase.

Likewise, the decay of the mean on relaxation from the value

$$\mu_{n,0} = (i/\sigma^2)[n\sigma_A^2 + \sum_{r=1}^n (b^{r-1})\frac{1}{2}\sigma_{AA}^2]$$

to

$$\lim_{t \rightarrow \infty} (\mu_{n,t}) \rightarrow (i/\sigma^2)n\sigma_A^2,$$

simulates the response which would occur if natural selection were operating in the absence of artificial selection to regress the mean toward its original unselected value.

Thus, if epistatic contributions are not taken into consideration, the disturbance they cause may be confounded with, or wrongly judged due to, the antagonistic effects of natural selection. Therefore, it is necessary to outline a possible method of detecting the influence of natural selection in the presence of disturbances caused by epistatic effects. Such a method will be given after the basic requirements for a selection programme aimed at detecting natural selection are given.

## (a) Requirements of a Selection Programme

The following lists the basic requirement in a selection programme designed to detect the effects of natural selection in opposing artificial selection:

- (i) Use of a truly random-mating population in equilibrium as the original population with which the selection programme starts;

- (ii) Collection of sufficient data to allow for the accurate estimation of parent-offspring and half-sib covariances in the original population (this requires a very large sample of observations);
- (iii) Collection of data from several cycles of truncation selection based on the individual phenotype (the selection must be conducted with known intensity and with large numbers of individuals); and
- (iv) Collection of data from selected lines which have been allowed to mate at random for several generations, i.e. from relaxed lines.

There are other experimental ramifications which are useful. These include the following:

- (v) Simultaneous selection experiments conducted with the same intensity in opposite directions;
- (vi) Simultaneous selection experiments conducted with different intensities of selection;
- (vii) Relaxation of selection at various stages in a continuous selection programme;
- (viii) Back-selection of relaxed lines after they have become stabilized;
- (ix) Mass reciprocal crossing of highly selected *relaxed* lines; and
- (x) Sufficient fitness measurements to give a developmental picture of the change in fitness throughout the selection programme.

These subsidiary experimental procedures give additional information, allow a broader basis for the comparison of experimental results with selection theory, and provide some measure for the verification of the validity of the assumptions on which the theory is based. For example, simultaneous selection in opposite directions provides a useful check on the fulfilment of the assumptions, since if the assumptions hold, a symmetrical response should be obtained by the application of identical "up" and "down" selection pressures. If an asymmetrical pattern develops, at least one of the assumptions has been violated.

#### (b) *A Method for Detecting the Effects of Natural Selection*

When data from a selection programme, as outlined above, are available, the influence of natural selection may be detected, in the presence of epistatic disturbances, by comparing the observed responses due to artificial selection with those expected from the theory presented earlier which assumes no disturbance from natural selection. This requires the computation of accurate, unbiased estimates of  $\sigma_A^2$  and  $\sigma_{AA}^2$  from the original unselected population and replacing the variances in the selection formulae with these estimates. If the observed means do not differ significantly from the expected values, it may be inferred, generally, that natural selection does not have an appreciable effect. If, however, the observed means are significantly less than the expected values, it may generally be assumed that natural selection is opposing the effects of artificial selection. This argument holds only if the assumptions inherent in the theory have not been violated.

## VI. DISCUSSION

This paper deals primarily with the extension of mass selection theory to accommodate linkage. The various aspects of this problem are developed in three stages.

(i) Mass selection theory for two loci is extended to accommodate different recombination values for the two sexes. This theory is now completely general in that it permits: (1) any number of alleles at each locus; (2) arbitrary linkage, including the condition that the recombination value may be different for the two sexes; and (3) arbitrary dominance and epistatic effects.

This extension of the two-locus theory is necessary if one is to take into consideration the general phenomenon of linkage in organisms such as *Drosophila* which have different recombination values in the two sexes.

(ii) A method is developed for estimating the recombination value averaged over all possible pairs of loci scattered at random over all chromosome sets.

The solution to this problem is necessary in order to adapt the generalized two-locus theory, developed in the first stage, to cope with genetic situations which are considerably more complex. That is to say, the solution to stage (ii) allows the variability generated by a genetically complex system involving many loci to be approximately described by the relatively simple two-locus theory.

(iii) The expectations of the half-sib and full-sib covariances for a random-mating population are generalized to permit different recombination values for the two sexes.

Solution to stage (iii) is necessary for the estimation of genotypic variance components from covariances between relatives which are, themselves, subject to linkage disturbances. These variance components can then be used for the purpose of comparing observed with theoretical selection responses.

Finally, the above theory is used to outline a method which permits the detection of the influence of natural selection in modifying the effectiveness of artificial selection even when the mimicking effects of epistasis are present.

Clearly, the approach to the linkage problem adopted in this study results in only an approximate solution, since various simplifying assumptions are required. These include: (1) epistatic interactions involving three or more loci are negligible, and (2) the true value of  $\text{Cov}_{(m)}(\text{HS})$  can be approximated by the  $\text{Cov}_{(m)}(\text{HS})$  derived from the two-locus model in which the average recombination value is substituted for the specific value for the two loci.

The usefulness of the theory rests on its ability to describe observed selection results. At the present time, the only data available which are sufficiently comprehensive to make this comparison are those reported by Clayton, Morris, and Robertson (1957) and Clayton and Robertson (1957). Unfortunately, however, the basic covariances from the original population were not estimated with sufficient accuracy to provide meaningful estimates of the variance components. Therefore, comparison of observed responses with the theoretical results of the present study will have to be made in the future when sufficiently accurate data are available.



## VII. ACKNOWLEDGMENTS

I am grateful to my colleague, Dr. R. N. Oram, for his valuable suggestions regarding the crossing-over argument.

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# THE EFFECTS OF SUB-ZERO TEMPERATURES ON THE EMBRYONIC DIAPOUSE OF *ACHETA COMMODUS* (WALK.) (ORTHOPTERA)

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## Summary

The effects of temperature on the termination of diapause in eggs of *Acheta commodus* were investigated over the range of  $-16.5$  to  $+12.8^{\circ}\text{C}$ ; the effects on the entry into diapause were also measured by exposing pre-diapause eggs to temperatures of  $-10.0$  to  $+12.8^{\circ}\text{C}$ .

The pre-diapause eggs were killed by temperatures below  $10^{\circ}\text{C}$  at periods of exposure insufficient to completely eliminate diapause. The lower the temperature the higher was the proportion of eggs killed. This sensitivity of pre-diapause eggs to low temperatures is considered to be the reason for the high optimum temperature previously recorded for the elimination of diapause by this method.

These limitations did not apply to eggs in diapause which were able to withstand the exposures necessary for the termination of diapause at sub-zero temperatures sufficiently well to enable the rate of termination of diapause to be determined down to  $-16.5^{\circ}\text{C}$ . Nevertheless, mortality was an important factor in these experiments and was responsible for the lower percentage hatching which occurred in the sub-zero treatments as compared with above zero. Melanization occurred at temperatures below  $-5^{\circ}\text{C}$ , but this was not necessarily an indication of mortality.

The rate of termination of diapause was found to be highest at sub-zero temperatures. Moreover, a negative temperature coefficient was obtained from  $+5^{\circ}\text{C}$  down to  $-16.5^{\circ}\text{C}$ , so that, in these particular tests, whereas at  $5^{\circ}\text{C}$  the median effective duration of exposure was 17 days, 8 hr were required at  $-5^{\circ}\text{C}$ , and only 20 min at  $-16.5^{\circ}\text{C}$ . The actual value of the median effective duration of exposure varies from one batch of eggs to another.

Above  $5^{\circ}\text{C}$ , there was a slight increase in the rate of termination at  $10^{\circ}\text{C}$  and a decline at  $12.8^{\circ}\text{C}$ , so that  $10^{\circ}\text{C}$  was the optimum for temperatures above zero.

The results with *Acheta* are exceptional, therefore, not only in the effectiveness of sub-zero temperatures for the termination of diapause in an insect from a mild climate, but also because the rate of termination showed a negative temperature coefficient from  $-16.5$  to  $+5^{\circ}\text{C}$ .

## I. INTRODUCTION

Many species of insects overwinter in a state of diapause, the termination of which is most commonly brought about by a period of exposure to low temperature; the necessary period varies with the species and the temperature.

Despite variations, the low-temperature requirements of different species are sufficiently uniform in certain characteristics to suggest that the underlying processes, at least in a high proportion of species, are the same.

Such a conclusion is implicit in the more recent reviews of the subject, in that generalizations are sought on the factors that the different species have in common with respect to the temperature requirements for the termination of diapause.

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Thus, in relation to the most favourable level of temperature for the termination of diapause, Andrewartha (1952) stated that "although much more information would be required before a generalization could be made it seems likely that the typical relationship . . . may be expressed as a sigmoid curve which leaves the axis somewhere about 0°C, reaches a maximum not far below the lowest temperature for morphological development and declines . . .". Lees (1955), in his review of the subject, agrees with this conclusion and also with Andrewartha's concept of diapause as a developmental process "influenced by temperature in much the same fashion as morphogenesis", i.e. that a positive temperature coefficient exists over the most favourable section of the range.

Although the requisite temperatures are commonly within a restricted range, the most effective portion of this range varies from one species to another and appears to be related to the climatic zone occupied by each species in such a way that the warmer the climate the higher will be the optimum temperatures for the rate of termination of diapause. Danilyevsky (1949) seems to have been the first to elaborate on this viewpoint; Lees (1955) reached similar conclusions, and Masaki (1959) refers to this relationship as an ecological rule.

Previous work on *Acheta commodus* indicated conformity of diapause in this insect with the above principles (Browning 1952). However, *Acheta* has the unusual characteristic that exposure of pre-diapause eggs to a temperature of about 13°C so weakens the tendency of the eggs to enter diapause that it is readily averted when they are transferred to a suitable incubation temperature. This response Browning attributed to the completion of diapause development concurrently with morphogenesis. Actually low temperature may be said to eliminate diapause from such eggs rather than to terminate it.

If diapause development is completed in the pre-diapause stages, then there should be a marked similarity between the relative effectiveness of different temperatures for the elimination of diapause from pre-diapause eggs, and for the termination of diapause in eggs that have actually entered diapause. Thus the optimum temperature and the general form of a curve representing the effectiveness of different temperatures should be the same in each case.

Therefore, although the present experiments were concerned primarily with the effective and optimum temperatures for the termination of diapause, pre-diapause eggs were also tested in order to enable a comparison of the above values. The results are discussed in relation to the above principles and the evidence they provide as to the nature of the diapause mechanism.

## II. METHODS AND MATERIALS

The eggs used in the experiments were from crickets (*Acheta commodus* (Walk.)) reared in the laboratory on a standardized diet and at a temperature of 29.4°C.\* The previous generation of crickets had been collected from the field.

After oviposition at 26.7°C the eggs were transferred to a cabinet at 23.3°C where they were held for 14–16 days, this period being necessary to fully induce

\* All temperatures quoted were controlled to within  $\pm 0.2^\circ\text{C}$ , unless otherwise indicated.

diapause. For the experiments on pre-diapause eggs, oviposition trays were left in the cages for 16 hr, after which the eggs were sieved out and placed at the appropriate low temperature.

During the temperature treatments both the pre-diapause and diapause eggs were held in plastic tubes at the base of which was a disk of moistened blotting-paper. These tubes were held in plastic boxes and both the tubes and the boxes were left without lids during the exposure to low temperature and sealed during the incubation at 26.7°C. Prior to each treatment, the diapause eggs were held at 12.8°C for several hours to reduce the extent of temperature change. In the control treatments both the diapause and pre-diapause eggs were incubated at 26.7°C.

The number of cabinets available for low-temperature tests were insufficient to cover the full range of treatments; hence it was necessary to carry out a series of tests linked by treatments in common. The controls provided evidence as to the comparability of the different batches of eggs. While some of the data were not particularly suitable for the determination of median points, they nevertheless permit of quite clear-cut conclusions.

The eggs were incubated for 15 days at 26.7°C following the low-temperature treatments. Any that did not hatch were held at this same temperature until they showed evidence either of resumption of development or of mortality. This was found necessary because eggs that appeared healthy and in diapause were found on microscopical examination to show abnormalities of the embryo. Such eggs did not resume development even after prolonged incubation and were assumed to be dead. Apparently diapause conferred on them a resistance to breakdown even after death.

The term "median effective duration of exposure" (M.E.D.E.) used by Browning (1952) to denote the period of exposure necessary to enable resumption of development by 50 per cent. of the eggs has been adopted here. It should be noted that the percentage hatching and, therefore, the M.E.D.E. will be dependent on the incubation temperature; the latter was 26.7°C throughout these experiments. The period required to cause 50 per cent. mortality has been termed the "median lethal duration of exposure" (M.L.D.E.). The rate of termination of diapause has been expressed as the reciprocal of the M.E.D.E.

The pre-diapause treatments unavoidably included a proportion of non-viable eggs since these were indistinguishable from viable eggs during the first 16 hr after oviposition. Such eggs were not present in the diapause treatments as they were readily distinguishable by their failure to take up moisture during the diapausing process and were rejected.

The term "elimination of diapause" has been used to denote the process described in the Introduction whereby the onset of diapause is prevented. Diapause is not finally eliminated until the eggs are incubated at a higher temperature (Hogan 1960).

### III. RESULTS

#### (a) *Pre-diapause Eggs*

Pre-diapause eggs, not more than 16 hr after oviposition, were held at temperatures of -10, -7.5, 0, 5, 10, and 12.8°C; three tubes with 25 eggs per



tube were used for each treatment except at  $-7.5$  and  $-10^{\circ}\text{C}$ , in which the treatments were more closely spaced and single tubes with 30 eggs each were used. The control treatments were held at a constant temperature of  $26.7^{\circ}\text{C}$  and indicated the proportion of eggs not subject to diapause at this temperature. It also served as a measure of the uniformity of the eggs.

TABLE 1

PERCENTAGE OF VIABLE EGGS THAT HATCHED AFTER LOW TEMPERATURE DURING THE PRE-DIAPAUSE STAGES WITH INCUBATION AT  $26.7^{\circ}\text{C}$ , AND THE PERCENTAGE OF THE ORIGINAL NUMBER OF EGGS THAT DIED AFTER TREATMENT

Temp. (°C)	Period of Exposure (days)	Hatching (%)	Mortality (%)	Temp. (°C)	Period of Exposure (hr)	Hatching (%)	Mortality (%)
12.8	10	50.0	1.3	0.0	16	10.4	20.0
	17	73.2	5.3		24	2.2	23.3
	25	92.1	16.0		48	6.1	45.0
	38	100	5.3		120	—	90.0
	Control*	6.8	1.3		Control	13.5	13.3
10.0	10	31.4	6.7	−7.5	0.5	3.4	3.3
	17	53.6	8.0		1	37.0	10.0
	25	64.6	13.3		2	—	90.0
	38	100	26.7		4	—	100
	Control	6.8	1.3		Control	8.0	16.7
5.0	6	42.4	21.3	−10.0	0.25	0	0
	11	41.9	42.7		0.5	14.3	6.7
	25	—	100		1	17.6	43.3
	Control	27.9	9.3		2	—	96.7
					3	—	100
					Control	3.5	4.4

\* All controls were incubated at  $26.7^{\circ}\text{C}$  for 14 days.

At each temperature a series of treatments, with increasing periods of exposure, was tested. The durations of exposure were based on preliminary trials to determine the maximum period of time the eggs could tolerate at each temperature. The percentage viable hatching and the percentage mortality are shown in Table 1. In Table 2 the maximum observed percentage hatch at each temperature and the maximum percentage mortality have been corrected by Abbott's formula (Finney 1952):

$$100(P-C)/(100-C),$$

where  $P$  is the uncorrected percentage and  $C$  the percentage in the controls. The term "observed maximum hatch" has been used to indicate that the absolute maximum hatching has not necessarily been achieved in the durations of exposure chosen for the lower temperatures; onset of mortality can affect this value. In

view of the high mortalities obtained at these temperatures the absolute maximum hatch is not an important parameter in this experiment.

The extreme sensitivity of the pre-diapause eggs to low temperature is indicated by the M.L.D.E. values (Table 2). At 5°C, 50 per cent. of the eggs were killed in 14 days, while at -10°C a 63-min exposure was sufficient. This sensitivity to low temperature made it impracticable to measure the effect of the lower temperatures on diapause, since many of the eggs died before the diapause reactions were completed. Log (M.L.D.E.) appears to be linearly related to temperatures over the range -10 to +5°C and indicates that rate of kill (reciprocal of M.L.D.E.) is characterized by a constant (negative) temperature coefficient.

TABLE 2

EFFECTS ON PRE-DIAPAUSE EGGS OF EXPOSURE TO LOW TEMPERATURES FOLLOWED BY INCUBATION AT 26.7°C

Maximum observed percentage hatch and maximum percentage mortality are given after correction for controls by Abbott's formula

Temp. (°C)	Maximum Observed Hatch (%)	M.E.D.E.* (days)	Maximum Mortality (%)	M.L.D.E.†	Log (M.L.D.E.) (min)
12.8	100	11	15	—	—
10.0	100	17	26	—	—
5.0	20	—	100	14 days	4.3
0	0	—	88	55 hr	3.5
-7.5	32	—	100	‡	‡
-10.0	18	—	100	63 min	1.8

\* Median effective duration of exposure.

† Median lethal duration of exposure.

‡ Not satisfactorily established by the data.

Even at 10°C mortality became appreciable at the longer periods of exposure (25 days). This must be taken into account when comparing 10 with 12.8°C. At both these temperatures diapause was eliminated from all the viable eggs, but it took longer at 10 than at 12.8°C (17 compared with 11 days). This is the reverse of the result with diapause eggs where 10°C gave a higher rate of termination than 12.8°C (Table 3).

However, it would not be valid to assume that this indicates a difference in the nature of the processes involved in pre-diapause eggs as compared with diapause eggs. Since 5°C proved lethal to pre-diapause eggs, and the longer exposures to 10°C killed a proportion of the eggs, it seems likely that the shorter exposures to 10°C are unfavourable even though not lethal. Hence the resumption of development may be delayed in these eggs thereby giving a higher M.L.D.E.

It is concluded that the reason why 12.8°C was recorded previously as the optimum temperature for the elimination of diapause is because the rate of

elimination was measured by the rate of hatching, and 12.8°C was the lowest temperature, of those tested, at which there was no unfavourable effect on the viability.

TABLE 3

PERCENTAGE OF DIAPAUSE EGGS THAT HATCHED AFTER THE LOW-TEMPERATURE TREATMENTS SPECIFIED, AND THE PERCENTAGE OF THE ORIGINAL NUMBER OF EGGS THAT DIED AFTER TREATMENT

Temp. (°C)	Period of Exposure (days)	Hatching (%)	Mortality (%)	Temp. (°C)	Period of Exposure (days, except where indicated)	Hatching (%)	Mortality (%)
12.8	15	30.7	0	-5	3 hr	52.7	1.3
	30	76.0	0		7 hr	78.4	1.3
	43	93.2	1.3		16 hr	91.7	4.0
	61	100	1.3		1	91.9	1.3
	Control*	12.0	0		2	97.3	2.7
					5	95.9	2.7
10.0	15	42.7	0	-7.5	9	77.9	9.3
	30	90.5	1.3		15	40.5	50.7
	43	98.7	0		Control	51.4	1.3
	61	98.7	0		2 hr	46.7	0
	Control	12.0	0		4 hr	74.7	0
					7 hr	80.0	0
5.0	15	48.0	0	-10	1	89.0	2.7
	30	61.3	0		2	97.3	1.3
	43	81.3	0		5	69.0	22.7
	61	90.7	0		9	28.9	40.0
	Control	12.0	0		Control	51.4	1.3
					1	65.5	3.3
0.0	8	85.0	0	-16.5	1 hr	41.1	2.7
	12	76.9	13.3		3 hr	84.9	2.7
	16	79.3	51.7		7 hr	85.2	28.0
	20	100	75.0		16 hr	100	92.0
	30	0	100		1	0	100
	Control	8.5	1.7		2	0	100
				Control	51.4	1.3	
					5 min	0	—
					10 min	20.0	—
					15 min	26.7	—
					20 min	53.3	—
					Control	38.7	—

\* All controls were incubated at 26.7°C for 14 days.

### (b) Diapause Eggs

Eggs were brought into diapause by transferring them, when not more than 16 hr old, to a temperature of 23.3°C for 14–16 days. They were then counted into tubes: three tubes with 25 eggs in each were allotted to each treatment. The eggs

in the control treatment entered diapause in the same way as the other treatments and were then incubated at 26.7°C, thus providing a measure of the proportion of eggs able to hatch without exposure to low temperature.

The rate of termination of diapause was measured at eight temperatures ranging from -16.5 to +12.8°C and at each temperature the effects of various periods of exposure were tested. The rate of termination of diapause was derived from the measurement of the M.E.D.E. and calculated from the expression

$$\text{Rate} = 100/\text{time (days) for 50 per cent. hatch.}$$

Table 3 shows the percentage hatch and the percentage mortality for each treatment. In view of the extreme range of the values for the rate of termination these have been converted to log rate in Table 4, and are plotted against temperature in Figure 1.

TABLE 4

RATE OF TERMINATION OF DIAPAUSE AND THE TEMPERATURE COEFFICIENT FOR DIAPAUSE EGGS AFTER DIFFERENT LOW-TEMPERATURE TREATMENTS, BASED ON THE MEDIUM EFFECTIVE DURATION OF EXPOSURE (M.E.D.E.)

Temp. (°C)	M.E.D.E.	Rate of Termination*	Log Rate	Temperature Coefficient
12.8	22 days	4.50	0.65	-2.24
10.0	18 days	5.64	0.75	+1.39
5.0	21 days	4.79	0.68	-679.2
0.0	19 hr	124.6	2.10	-5.55
-5.0	8 hr	294.0	2.47	-8.60
-7.5	5 hr	503.8	2.70	-14.32
-10.0	2.5 hr	978.9	2.99	-20.23
-16.5	21 min	6923.0	3.84	

\* See Section III(b) for method of calculation.

It is clear that the outstanding result was the rapid rate of termination of diapause at sub-zero temperatures (Fig. 1). From 5°C down the temperature scale to -16.5°C the rate of termination of diapause progressively increased, i.e. the responses gave a negative temperature coefficient. The proportion of eggs terminating diapause at sub-zero temperatures was reduced to some extent by mortality at the longer exposures and the lower the temperature the higher was the mortality for the same period of exposure.

After treatment with temperatures above zero the rates of termination, derived from the M.E.D.E., were extremely low compared with those after sub-zero treatments (Table 4). However, the rate at 10°C was significantly higher than at 5°C, with a slight decline in effectiveness from 10 to 12.8°C. The form of the curve for temperatures above zero is, therefore, in accordance with that usually obtained for the termination of diapause in insects from a temperate climate, though the effect is not obvious in Figure 1 because of the scale used.

From the temperature coefficients (Table 4) and from Figure 1 the maximum increase of the rate of termination, over the whole range of temperatures tested,



appears to be associated with the 0–5°C interval. It should be noted, however, that the value for 0°C is not as well established as the other values owing to the relatively high hatching, 65 per cent., at the minimum period of exposure.

Below –5°C many eggs became blackened and this effect was more pronounced the lower the temperature to which the eggs were exposed. Melanization cannot, however, be taken as a sign of death; in some treatments the eggs became completely black, yet normal hatching occurred after incubation.

At some periods of exposure at each sub-zero temperature there was a proportion of eggs which did not hatch in the normal incubation time, but which hatched after an extended period of incubation. It is considered that these

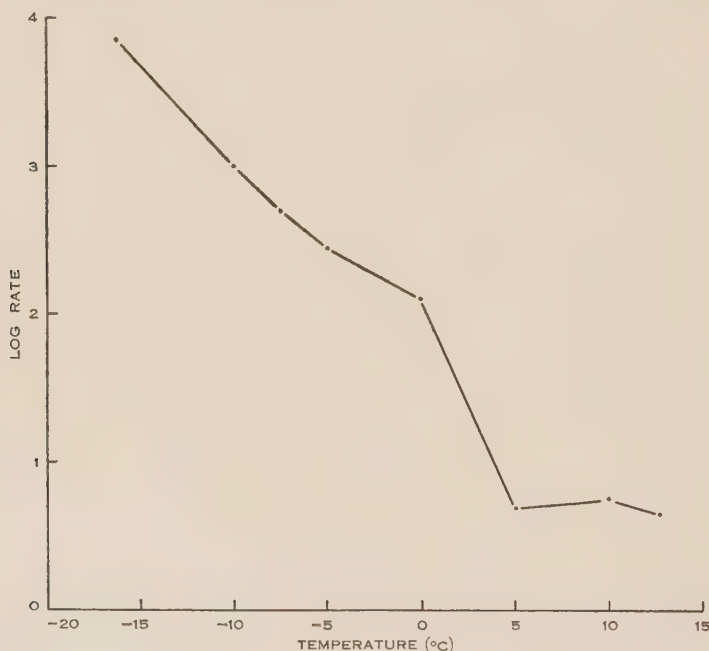


Fig. 1.—Effect of low-temperature treatments on the rate of termination of diapause, expressed as the log rate and based on the time taken for 50 per cent. of the eggs to hatch when incubated at 26.7°C.

represented eggs in which the normal metabolism was adversely affected and the delay in the resumption of development was a symptom of this effect.

Throughout these experiments, the termination of diapause and the occurrence of mortality were interlinked so closely that the interpretation of the results is, in some instances, restricted by the possibility that unfavourable effects on the rate of development, and the readiness to resume development, may occur. This applies more particularly to those treatments near the lethal dosage level, but which do not actually cause the death of the eggs.

Thus at 5°C, where the initial hatching was as high as that at 10°C, the subsequent hatching at the longer exposures was lower at 5°C (Table 3). It seems likely that this is due to the unfavourable effects of prolonged exposure at 5°C.

Different batches of eggs were found to vary in their sensitivity to low temperature, but the variations appeared to be independent of the strength of diapause (as measured by the control treatment).

A further aspect of the reactions of diapause eggs to low temperature requires emphasis. When treatments were lethal to pre-diapause eggs the effect was seen by such symptoms as discoloration, collapse, growth of mould, either in combination or separately. In diapause eggs, on the other hand, it was found that many eggs failed to resume development after the normal incubation period and retained the normal appearance of diapause eggs. However, if they were incubated for much longer periods, they did not resume development. Furthermore, if such eggs were cleared by the process previously described (Hogan 1959), the embryos appeared misshapen and abnormal. There can be little doubt, therefore, that the eggs had been killed by the treatment, but were more resistant to breakdown than eggs at other stages. If held long enough at the incubation temperature, they eventually showed the usual symptoms given by pre-diapause eggs that had been killed by low-temperature treatment.

#### IV. DISCUSSION

The results in the foregoing experiments differ substantially from those usually recorded for the termination of diapause in insects by low temperature.

In the first place, the responses of *Acheta* over the most favourable range give a curve with a negative temperature coefficient. This type of response is contrary to the generally accepted rule that over the most effective range of temperature for the termination of diapause the responses give a positive temperature coefficient.

Secondly, the results with *Acheta* are contrary to the rule that the warmer the climatic region to which the species belongs, the higher is the optimum range of temperatures effective for the rate of termination of diapause. *Acheta* comes from a region with moderate winters (see Fig. 3) yet the most effective temperatures are sub-zero.

Previous results with *Acheta* (Browning 1952) indicated conformity with both of the above principles. However, Browning's experiments were with pre-diapause eggs and, as is clear from the results in Section III(a), the influence of low temperatures on pre-diapause eggs are affected by the inability of pre-diapause eggs to survive the necessary period of exposure at sub-zero temperatures. Lethal effects also set a lower limit to the investigation on the effect of sub-zero temperature on diapause eggs.

#### *Termination of Diapause in other Species of Insects*

The principles underlying the termination of diapause have been discussed in reviews on the subject by Andrewartha (1952) and Lees (1955). The relationship between the climatic zone occupied by the insect and the conditions necessary for the termination of diapause were reviewed in rather more detail by Danilyevsky (1949). The general conclusions reached by Lees (1955, pp. 50-58) from data for 41 species of Arthropoda are in agreement with those of earlier authors and are taken to be a fair sample of the data on which existing conclusions are based. They are now re-examined in the light of the results obtained at sub-zero temperatures with *Acheta*.

Of the 41 species it was found that the reactions to sub-zero temperatures had been investigated in only nine. Table 5 shows that of these nine species sub-zero temperatures were effective in terminating diapause in six and ineffective in the other three. The three species for which sub-zero treatment was found ineffective were (1) the egg stage of the mite *Metatetranychus ulmi* (Lees 1953); (2) the larval stage of *Cephus cinctus* (Salt 1947); and (3) the egg stage of *Alsophila pometaria* (Flemion and Hartzell 1936).

TABLE 5  
EFFECT OF SUB-ZERO TEMPERATURES ON THE TERMINATION OF DIAPAUSE IN NINE SPECIES CITED BY LEES (1955)

Species	Stage	Reference	Minimum Temp. (°C)	Result*
<i>Alsophila pometaria</i> (fall cankerworm)	Egg	Flemion and Hartzell (1936)	-16	I
<i>Lymantria dispar</i> (gypsy moth)	Egg	Kozhantshikov (1950)	-2	E
<i>Malacosoma disstria</i> (forest tent caterpillar)	Egg	Hodson and Weinman (1945)	-5	E
<i>Metatetranychus ulmi</i> (European red mite)	Egg	Lees (1953)	-5	I
<i>Aporia crataegi</i> (hawthorn butterfly)	Larval	Zolotarev (1950)	-5	E
<i>Cephus cinctus</i> (wheat stem sawfly)	Larval	Salt (1947)	-20, -15	I
<i>Gilpinia polytoma</i> (European spruce sawfly)	Larval	Prebble (1941)	-10	E
<i>Antheraea pernyi</i> (Chinese oak silkworm)	Pupal	Zolotarev (1947)	-5	E
<i>Saturnia pavonia</i>	Pupal	Danilyevski (1949)	-15	E

\* I, ineffective; E, effective.

Since *Acheta* is an insect with embryonic diapause, it would appear advisable to restrict consideration to insects with embryonic diapause. This leaves only *Alsophila*. The eggs of this insect were exposed to a temperature of  $-16^{\circ}\text{C}$  for a minimum period of 3 weeks and up to a maximum of 16 weeks. Except for one egg after the 3 weeks period no hatching occurred, but as to whether the unhatched eggs were viable or not there is no indication.

Thus in these nine species, all of which are from colder climates, sub-zero temperatures were effective for the majority; however, in no cases were sub-zero temperatures the optimum.

As regards species from regions with moderate to warm winters, their responses to sub-zero temperatures do not seem to have been tested in any of the species quoted by Lees. In papers published since 1955 the above conclusions also apply, with one important exception (Way 1959, 1960), which will be discussed separately.

The absence of experiments in the sub-zero range with insects from warmer climatic zones is, presumably, due to the unsatisfactory results obtained at zero, or near zero, temperatures. It is of interest, in this regard, that if the most usual procedure for determining the effect of temperature on the termination of diapause is applied to *Acheta*, an artefact is produced by which 10°C appears to be the optimum temperature for the termination of diapause. Thus if the period of exposure is based on that required at 10°C (followed by a suitable incubation period), then a 45-day exposure would be deemed suitable. After such a treatment there would be no hatching at 0°C, some hatching at 5°C, and a high percentage hatching at 10°C (Fig. 2).

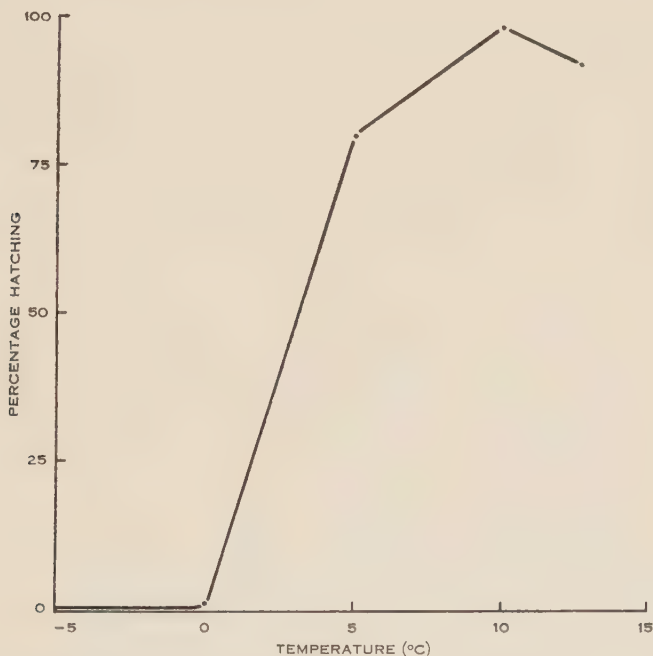


Fig. 2.—Rate of completion of diapause in eggs of *Acheta*, based on the rate of hatching, after 45 days at low temperature and incubation at 26.7°C.

The explanation of this anomaly lies in the fact that when the eggs are exposed to 0°C for a period sufficiently long to cause their death, they nevertheless retain their normal external appearance and would simply be included as eggs in which diapause had not been terminated.

No data are available by which to decide whether unobserved mortality could have been a factor in the low hatching reported for the species from cold climates following treatment at sub-zero temperatures. That such a possibility exists has been demonstrated by the reactions of *Leptohylemia coarctata* to freezing temperatures (Way 1960). This species inhabits a climatic zone considerably colder than *Acheta* (Fig. 3), but the reactions to low temperature are similar in one important respect, viz. there is a negative temperature coefficient for the rate of termination of diapause.



The threshold for this effect is at  $-6^{\circ}\text{C}$  for *Leptohylemia* (extending down to  $-20^{\circ}\text{C}$ ), whereas in *Acheta* the threshold is from  $+5^{\circ}\text{C}$  downwards. In each species the lower limit of effectiveness appears to be governed by the onset of mortality.

Both species, too, have a range of temperature over which there is a positive temperature coefficient strongly marked in *Leptohylemia* from  $-6$  to  $+10^{\circ}\text{C}$ , and rather weak in *Acheta*, from  $+5$  to  $+10^{\circ}\text{C}$ . Thus the effective temperatures are divided into two sections, the response on the lower side having a negative coefficient and those on the higher side having a positive coefficient. However, the intermediate point between these two divisions is different for the two species, being  $-6^{\circ}\text{C}$  for

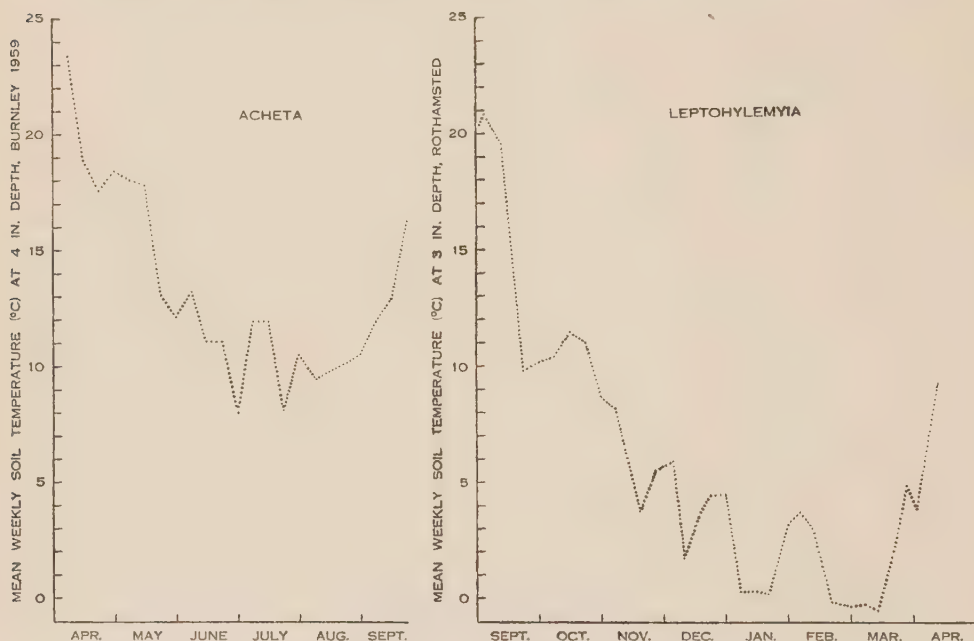


Fig. 3.—Mean weekly soil temperatures at a depth of 4 in. at Burnley, Vic., and those at 3 in. at Rothamsted, England (from Way 1960) over corresponding seasons, illustrating the milder winter experienced by *Acheta* as compared with *Leptohylemia*. (The difference in the depth of soil would affect the extent of the diurnal variations rather than the value of the mean temperature.)

*Leptohylemia* and  $+5^{\circ}\text{C}$  for *Acheta*. In addition the rate of termination of diapause at sub-zero temperatures, in comparison with those above zero, is much greater in *Acheta* than in *Leptohylemia*.

This suggests that there is an adaptation of the diapause of each species to suit the environment normally inhabited, in line with the previous ideas on the subject, but rather different in its expression.

It is clear that the results at sub-zero temperatures are of significance in relation to the nature of the diapause mechanism. Since the most effective temperatures are far below those normally experienced by *Acheta*, this reaction apparently represents a means whereby the end-point normally reached by "diapause development" at moderate temperatures, is achieved by another route.

In *Acheta* diapause ensues at an early stage in embryogenesis (Hogan 1960); in *Leptohylemia* it takes place when the embryo is fully developed, possessing a brain and an endocrine system (Way 1960), and is therefore regarded by Way as having more in common with post-embryonic than early embryonic diapause. If this be so, then the similarity in the reactions of the two species to sub-zero temperatures suggests that, basically, embryonic and post-embryonic diapause have the same mechanism.

The effectiveness of sub-zero temperatures recalls the early hypothesis that low temperature terminates diapause by the gradual destruction of an inhibitor. However, the results of these experiments, taken in conjunction with the previous ones (Hogan 1960) suggest that the reactions are more complex than could be accounted for simply by the presence of an inhibitor.

The temperature reactions below  $+5^{\circ}\text{C}$  with *Acheta* are also at variance with the concept of a developmental process, or biochemical reaction, since these would be expected to have a positive temperature coefficient.

The reactions are consistent with a hypothesis that a physicochemical change of state occurs whereby barriers to energy transfers, existing during the state of diapause, are removed.

#### V. ACKNOWLEDGMENTS

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# EXPERIMENTAL STUDIES ON POLE CELLS AND MIDGUT DIFFERENTIATION IN DIPTERA

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## Summary

Highly localized irradiation with ultraviolet of the posterior polar region of eggs of *Drosophila melanogaster* and *Lucilia cuprina* in pre-pole cell and pole cell stages results in reduction in numbers of the cuprophilic cells of the middle midgut as well as in reduction of gonad size and number. Carefully timed eggs were exposed to dosages of ultraviolet (from a source giving about 90 per cent. at wavelength 2536 Å) ranging from 1200 to 2400  $\mu\text{W sec/cm}^2$  over periods of 2–4 min. Treatments at the time of active pole cell formation were found to be most effective in producing defects of both gut and gonads, thus demonstrating the common origin of the cuprophilic cells of the middle midgut and the germ cells of the gonads.

Detailed quantitative data concerning gonads and midgut cells are presented for *D. melanogaster* for various ages at time of treatment. Separability of the types of defects before and after the time of active pole cell formation suggests the derivation of the cuprophilic cells from the more peripheral and the germ cells from the more internal polar regions. An inverse relation was found between the numbers of calycoocytes (cuprophilic cells) in the midgut epithelium and the numbers of free cells in the lumen of the gut in older embryos. Evidence indicates that larvae with severely reduced numbers of cuprophilic cells seldom survive either in *Drosophila* or *Lucilia*. Although the gut epithelium is usually continuous even in complete absence of cuprophilic cells, morphogenesis of the middle midgut is always abnormal in such embryos and larvae fail to hatch. Quantitative data are presented for surviving larvae of *Lucilia*.

A study of control embryos of *D. melanogaster* demonstrated sexual dimorphism with respect to calycoocyte number in the midgut epithelium at the time of hatching, the numbers being consistently higher in females than in males, an inverse correlation with gonad size.

## I. INTRODUCTION

Modern work on the physiology of the insect gut has demonstrated the presence of remarkable patterns of functional and morphological differentiation (Waterhouse and Day 1952; Waterhouse 1957). In particular, the larval midgut of Diptera provides excellent material for study and in *Lucilia cuprina* (Wied.) it has been demonstrated that, in addition to regional differentiation, there is differentiation among cells within regions (Waterhouse 1945; Waterhouse and Stay 1955). Thus the copper-accumulating cells of *L. cuprina* are distributed in a mosaic fashion

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among the lipoid cells of the middle midgut. In species of *Drosophila*, the cuprophilic cells of the larval midgut usually form a distinct region within which other cell types are either rare or relatively inconspicuous, although occasional instances of a mosaic arrangement may sometimes be found (Poulson and Bowen 1952; Waterhouse and Stay 1955; Poulson, unpublished data). In contrast to *Lucilia* and other related genera, the principal cuprophilic cells of *Drosophila* are morphologically quite distinct from other midgut cells. They are the goblet-like calycoocytes described by Strasburger (1932).

The remarkable properties of the cuprophilic cells, together with the fact that their cytological fine structure differs markedly from that of other gut epithelial cells (Waterhouse and Wright 1960), have led us to enquire into their embryological origin and the course of their differentiation.

The rationale of the experiments reported here rests on the observational evidence that only a fraction of the pole cells of the higher Diptera contribute to the germ cells of the gonads (Noack 1901; Rabinowitz 1941; Poulson 1947, 1950; Sonnenblick 1950), the balance, in *Drosophila* at least, becoming associated with the midgut rudiment (Poulson 1947, 1950). The technique employed is an adaptation of that used by Geigy (1931) in his classical demonstration that the germ cells of the gonads of *Drosophila* are derived from the pole cells. However, we have followed the effects of treatment on both the midgut and the gonads in embryos and larvae rather than confining observations to the surviving adults.

This paper provides an account of a number of experiments in which the pole cells and polar regions of the eggs of *L. cuprina* and *D. melanogaster* Meig. were exposed to ultraviolet radiation with the object of interfering with this region and the cells derived from it. A preliminary presentation has been given by Poulson and Waterhouse (1959).

## II. METHODS

Egg deposition, exposure to ultraviolet radiation, and subsequent incubation were carried out in a room maintained at 25°C and 75–80 per cent. relative humidity, thus minimizing disturbances from temperature and humidity changes.

### (a) Cultures and Egg Collection

Eggs of *D. melanogaster* were collected from young gravid females of the strain Oregon-R-C maintained in this Laboratory. Slides bearing a little cornmeal, molasses, and agar medium baited with honey and yeast were placed in the laying bottles for periods of a few minutes. Eggs of known age were harvested into saline for further use.

A culture of the Australian sheep blowfly, *L. cuprina*, containing many gravid females was offered liver slices and observed until egg laying commenced. Five minutes or less sufficed for the deposition of enough eggs for any one experiment. These were transferred to moist filter paper (since immersion in saline for any period produced adverse effects) until the appropriate time for treatment.

(b) *Dechoriation*

*Drosophila* and *Lucilia* eggs at 30 min or more after laying (depending on how early it was desired to irradiate them) were immersed in a solution of sodium hypochlorite diluted with insect saline to a concentration (3 per cent.) at which dechoriation was accomplished in 1–2 min at 25°C. As this process was nearing completion the solution was drawn off and replaced by five or more successive lots of saline until all traces of hypochlorite had been removed. The eggs were then pipetted on to coloured blotting-paper to await transfer for irradiation. The moist substrate and high room humidity prevented desiccation. As a further precaution the heat rays from the microscope lamp used during manipulation were filtered off with Chance O.N.20 glass.

(c) *Irradiation*

The general technique used by Geigy (1931) and Aboim (1945) was adopted, although a number of modifications were introduced in order to increase the uniformity of the conditions of exposure. Dechorionated eggs were transferred with a glass needle to a rectangular coverslip which was very lightly lubricated with 50 per cent. aqueous glycerol to facilitate their manipulation into a straight line. Each egg was so oriented that it lay on its slightly convex ventral surface with the posterior pole at the edge of the coverslip. No difficulty was experienced with the orientation of *Drosophila* eggs. However, in *Lucilia* there was occasional difficulty in distinguishing the posterior pole of the egg prior to appearance of the pole cells. The egg-bearing coverslip was then lightly attached with vaseline to a brass wedge, thereby giving the eggs an inclination of 30° and making the posterior pole visible from vertically above (Figs. 1(b), 1(c)).

The brass wedge was, in turn, held in a tray about 1 mm deeper than it. The entire assembly was placed on a track which allowed the tray to be moved from under a dissecting microscope at one end to a point directly beneath an ultraviolet lamp at the other (Figs. 1(a), 1(b)). Two glass microscope slides (3 by 1 in.), rendered opaque with photographic masking paint, were used to form a narrow slit of determined width. After placing the wedge in the tray the first microscope slide was moved into position against a stop. The wedge was then moved with forceps under the microscope by means of the projecting pins (*P*, Fig. 1(c)) so that the same measured amount of the posterior pole of each egg protruded from under the opaque slide. The second opaque slide was then adjusted to give a parallel slit of desired width (Fig. 1(b)). The effectiveness of this shielding and the width of the slit were tested photographically.

For exposure to ultraviolet radiation the tray was moved along the track to a position directly beneath and 10 cm below an appropriately shielded ultraviolet source and a shutter opened for the desired period. The source\* was a coiled quartz tube containing neon, emitting 85–95 per cent. of its radiant energy at 2536 Å and producing, at 10 W, an intensity of  $10^4$  ergs/cm<sup>2</sup>/sec at 10 cm distance (Lea 1946). Exposure times ranged from 1 to 4 min. In most cases a 2-min exposure was employed; in others 3 min; and in one series a 4-min exposure was given to test dosage effects.

\* Thermal Syndicate Ltd., London.

In all the experiments reported here the slit width was constant (2 units on the eyepiece micrometer) as was the length of the portion of the posterior pole of the egg exposed (2 units). After exposure, the coverslip with eggs was transferred to a

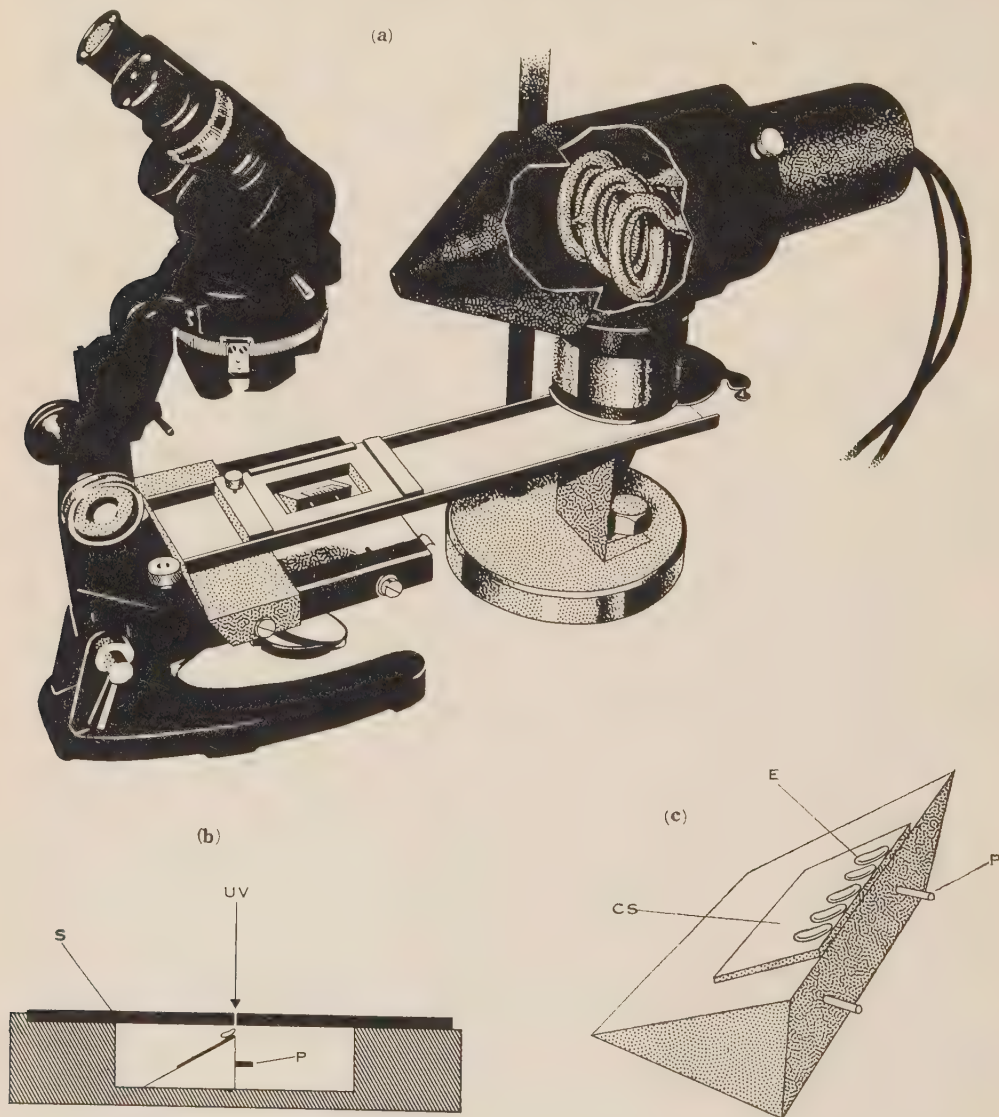


Fig. 1.—Apparatus used for irradiation: (a) tray with oriented eggs on holder is set on a track along which it is moved from the binocular microscope to a position directly beneath the source mounted over the track. (b) Details of tray with wedge and slit-forming slides (*S*) arranged for irradiation of pole of egg. (c) Wedge is moved with aid of pin (*P*); eggs (*E*) oriented on coverslip (*CS*) prior to alignment beneath slit.

loosely closed container lined with moist "Kleenex" tissue and incubated at 25°C in the dark to minimize photoreactivation (Altenburg and Altenburg 1952).



(d) *Histological Methods*

Eggs and newly hatched larvae were fixed by puncture with a fine glass needle in a formol-alcohol-acetic acid mixture (5 : 15 : 1 v/v) diluted to 50 per cent. with insect saline. After dehydration they were cleared in benzol and embedded in paraffin at 56°C, sectioned at 7  $\mu$ , and stained in Harris's haematoxylin. In a few instances sections of *Lucilia* eggs were cut at 9  $\mu$ .

The cuprophilic cells of *Lucilia* larvae were made distinguishable for counting by placing the freshly dissected midguts in a solution of neotetrazolium chloride as described by Waterhouse and Stay (1955).

(e) *Controls*

These consisted of embryos and larvae carried through all procedures except the irradiation and prepared as described.

### III. EXPERIMENTAL RESULTS

The experiments were carried out in three series. In the first the surviving larvae from treated eggs (of both *Lucilia* and *Drosophila*) were raised up to a stage at which the midgut cells could readily be recognized and counted using appropriate procedures. In these instances evidence of some reduction in the numbers of cuprophilic cells in larval guts of *Lucilia* was observed. However, it was at once apparent that these larvae represented only a portion of the treated eggs and that those which failed to hatch or to grow to normal size constitute the really significant material. Sections of these were then examined and evidence of gut and gonad disturbances were found. The data from these are presented along with those from a second series in which all embryos and larvae were fixed at the time of hatching. A third series consisted of embryos fixed at intervals of 30, 60, 90 min, and at 5 and 7 hr following treatment. These were useful in demonstrating the degree of localization and the more immediate effects of the radiation treatment.

Because of its larger size, the egg of *Lucilia* lends itself well to manipulation and allows more precision in localization of treatment; however, the lack of criteria for recognizing the cuprophilic cells in sections of embryos and early larvae makes quantitative work difficult. On the other hand, the principal cuprophilic cells of *Drosophila* (calycocytes) are morphologically distinguishable from other gut cells (Plate 1, Figs. 1-3) well before the time of hatching of the larvae and can be counted with relative ease. Hence we have used the *Drosophila* material for examining the quantitative relations following pole-cell treatment and the *Lucilia* material largely for comparative purposes and for following the localization and immediate effects of treatment.

(a) *D. melanogaster*—*Quantitative Relations*

Sections of treated *Drosophila* eggs fixed at the time of hatching of normals were carefully examined and counts made of the number of calycocytes in the middle midgut and of the number of gonads (and cells per gonad) in embryos for each time of treatment (Table 1). In a portion of the material the numbers of "large" cells (Fig. 2) in the region of the middle midgut just posterior to the calycocytes



were counted (Fig. 5, Table 5). In unhatched larvae the numbers of "free" cells in the lumen of the gut were also counted. The relationships between these and calycoocyte numbers are given in Figure 4. The data presented include only embryos, all sections of which were complete and analysable for the different ages at treatment.

TABLE 1  
NUMBERS OF CALYCOCYTES AND GONADS IN TREATED EMBRYOS OF *D. MELANOGASTER* FOR DIFFERENT AGES AT TIME OF TREATMENT

60-75 Min		79-86 Min		89-102 Min		103-119 Min		120-132 Min		124 Min	
Calyco- cyte	Gonad	Calyco- cyte	Gonad	Calyco- cyte	Gonad	Calyco- cyte	Gonad	Calyco- cyte	Gonad	Calyco- cyte	Gonad
74	2	76	0	60	0	65	0	82	1	72	2
73	0	76	2	60	2	62	2	75	2	60	2*
66	2	72	1	56	0	62	2	68	0	58	1
60	2	72	2	48	1	60	1	68	1	45	0
60	2	68	2	46	0	42	1	67	1	40	0
58	0	67	0	46	0	40	0	60	2	34	0
57	2	62	2*	42	0	38	2	60	2	32	2*
56	1	60	0	36	1*	36	0	60	2	23	0
55	1	56	2	32	0	35	1	58	2	22	0
54	2	56	0	32	0	34	2	56	2	14	0
54	2	51	2	32	0	30	0	51	1	0	0
54	0	50	0	30	0	28	0	40	2*		
54	2	48	2*	28	0	25	0	38	0		
52	0	40	2	24	0	20	0	26	0		
50	2	36	1*	24	0	18	0				
48	0	36	2	24	0	16	2				
42	2	14	2	20	0	0	0				
42	2	12	2	16	0	0	0				
38	0	7	0	14	0						
37	0	6	0	10	0						
34	0	0	0	6	0						
30	1	0	0	6	0						
0	0	0	0	3	0						
0	2	0	0	0	0						
0	0			0	0						
0	0			0	2						
				0	0						

\* Gonads in which germ cell numbers were much reduced.

(i) *Calycoocytes*.—The quantitative data on the effects of ultraviolet treatment on the middle midgut and the gonads are summarized in Tables 2 and 3 according to age at time of treatment. In Table 2 the mean numbers of calycoocytes per embryo are given in column 4. Calycoocyte numbers are markedly reduced in all groups as compared with the controls in which the mean was  $71.8 \pm 2.7$  with a range of 62-83. The proportions of embryos showing calycoocyte reduction are large throughout the series. Altogether 101 embryos out of 120 analysed showed reduction of calycoocyte number in the middle midgut.

That these may be considered as the minimal effects on calycoocytes will be evident by reference to Table 5 in which the numbers of calycoocytes in control

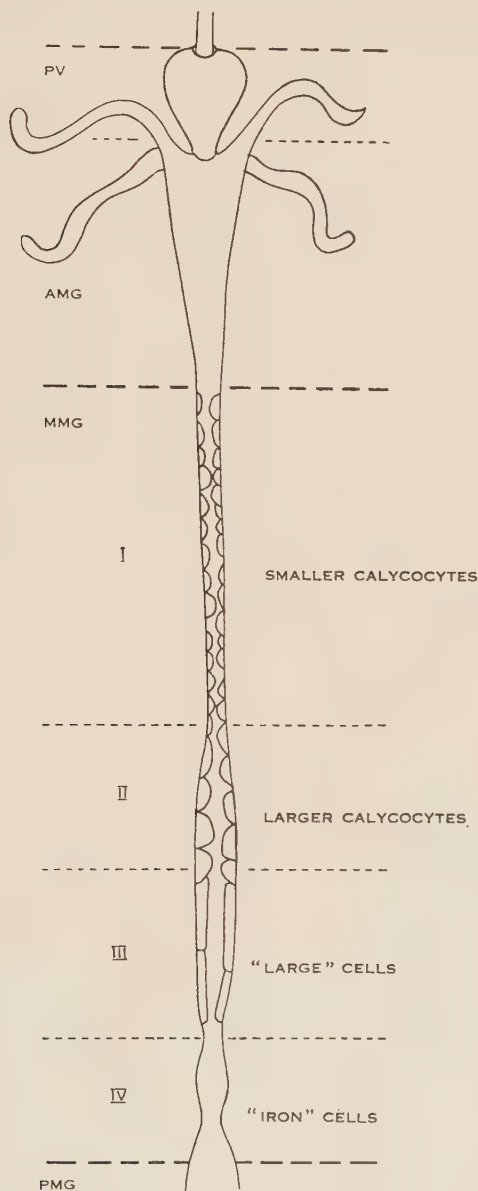


Fig. 2.—Diagram of a portion of the midgut of *D. melanogaster* indicating positions of calycoocytes of the middle midgut (MMG) relative to the anterior midgut (AMG) and the posterior midgut (PMG). The "large" cells described in the text form the epithelium in region III between the larger calycoocytes of region II and the "iron" cells of region IV.

embryos are presented along with data bearing on the sex of the embryos. In half these embryos the number of calycoocytes is between 70 and 83, in the others between

TABLE 2  
SUMMARY OF EXPERIMENTAL DATA ON CALYCOCYTE AND GONAD NUMBERS COMPARED WITH CONTROLS IN D. MELANOGASTER

Age at Treatment (min)	Dose (min)	No. of Embryos Analysed	Mean No. of Calycoocytes	Range of Calycoocyte Number	No. of Embryos with 60 Calycoocytes or Less	Mean No. of Gonads per Embryo	No. of Embryos with Gonads Affected	Embryos Herniated (%)
60-75	3	26	$44.1 \pm 4.3$	0-74	23	0.96	14	3.8
79-86	3	24	$40.2 \pm 5.7$	0-76	17	1.00	15	8.3
89-102	2, 3	27	$25.7 \pm 3.7$	0-60	27	0.22	25	44.4
103-119	2	18	$33.9 \pm 4.6$	0-65	15	0.72	13	33.3
120-132	2	14	$57.8 \pm 4.0$	26-82	9	1.28	8	0
124	4	11	$36.4 \pm 6.5$	0-72	10	0.63	10	18.1
Controls	0	12	$71.8 \pm 2.7$	62-83	0	2	0	0

62 and 70. By the criteria used, a reduction of calyccocyte number in one of the former to the range of the latter would be undetectable although it is highly probable that such must have occurred. The numbers of embryos involved are too small for the sort of statistical analysis which would be required to demonstrate this.

(ii) *Gonads*.—The effects on the gonads are also strikingly shown in Tables 1, 2, and 3. Most common is the complete absence of the gonads and germ cells. It was usually not possible in such instances to determine whether a gonad sheath was present and it seems probable, as discussed below, that the formation of a sheath is dependent on the presence of at least one pole cell in the appropriate part of the mesoderm. Cases of single gonads (unilaterals) were less frequent, there being 15 compared with 66 totally lacking gonads. However, these 81 do not represent the whole effect for in a number of instances germ cell numbers in gonads were much reduced. Five clear cases of this were recorded (marked with an asterisk in Table 1) among embryos with two gonads. Of these, four were among embryos with reduced calyccocyte numbers, the other was in an embryo with normal calyccocyte number. In these cases the number of cells per gonad was either 2 or 3 compared with the normal range (6–8 per gonad in females and 10–12 per gonad in males up to 18 hr; and higher numbers after the onset of pre-hatching mitoses—cf. Table 5). In only one group was the mean number of gonads per embryo above 1.00.

(iii) *Calyccocyte-Gonad Correlation*.—While correlation between calyccocyte and gonad reduction is apparent in Table 2 this is dramatically brought out in Table 3 in which the numbers and proportions of embryos affected (at different ages of treatment) with respect to *both calyccytes and gonads, calyccytes alone, gonads alone, and neither* are compared. This table was prepared from the data of Table 1 by summing up as affected for the different ages of treatment, all embryos with fewer than 60 calyccytes, and all with one or no gonad, together with the five cases of reduction of cell number in gonads described above. Thus, although calyccocyte reduction is greater, the gonad effects parallel the calyccocyte effects. The proportions of embryos in which calyccytes alone are affected are small. The proportion of unaffected embryos is generally less than 10 per cent. and we suspect that these escaped irradiation by virtue of being slightly misplaced in relation to the exposure slit.

To demonstrate the degree of correlation of the calyccocyte and gonad effects, the mean numbers of missing calyccytes and gonads were determined from Table 2 for the different age groups and plotted to give Figure 3.

(iv) *Calyccytes and Free Cells*.—A feature of a large proportion of treated embryos was the presence in the lumen of the midgut of numbers of free cells clearly distinguishable from yolk cells (*F*, Plate 2, Figs. 2 and 3). These cells were generally very similar in size to pole cells and early calyccytes and clearly represented cells which have failed to enter the midgut epithelium. As Table 5 shows, such free cells are rarely present in normal embryos. The emptying of the gut in newly hatched larvae soon removes all traces of these as well as the yolk remnants. Counts of free cells could only be made in embryos or unhatched larvae. The results for the different ages at time of treatment are presented in Figure 4 in relation to the numbers of calyccytes present. An inverse relation between numbers of free cells



TABLE 3  
RELATIONS BETWEEN CALYCOCYTE AND GONAD EFFECTS IN D. MELANOGASTER EMBRYOS OF DIFFERENT AGES AT TREATMENT

Cells Affected	60-75 Min		79-86 Min		89-102 Min		103-119 Min		120-132 Min		124 Min		Totals	
	No.	Fraction	No.	Fraction	No.	Fraction	No.	Fraction	No.	Fraction	No.	Fraction	No.	Fraction
Calycocytes and gonads	13	0.50	11	0.45	25	0.93	12	0.67	4	0.29	10	0.91	75	0.63
Calycocytes only	10	0.38	6	0.25	2	0.07	3	0.17	5	0.35	0	0	26	0.22
Gonads only	1	0.04	4	0.17	0	0	1	0.05	4	0.29	0	0	10	0.08
Neither affected	2	0.08	3	0.13	0	0	2	0.11	1	0.07	1	0.09	9	0.07
Totals	26	1.00	24	1.00	27	1.00	18	1.00	14	1.00	11	1.00	120	1.00

and calycoytes is apparent in all instances and is marked where older embryos (Figs. 4C, 4D) were treated. In the younger group (60–75 min together with 79–86 min) the relation appears roughly hyperbolic. During the period of pole cell formation (89–102 min) there is a wider scattering of points (Fig. 4B). In the next two groups, in which pole cell mitoses are numerous (103–119 min and 120–132 min), the linear relation becomes marked. In Figure 4D the 124-min age group of (double dose) embryos are included along with those receiving only the 2-min dose. Taken alone these show a clear inverse linear relation.

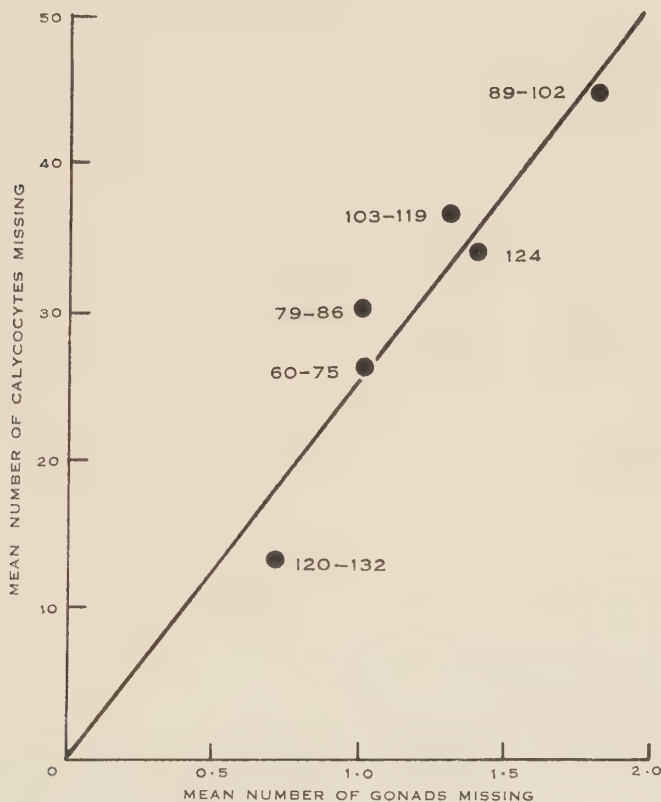


Fig. 3.—Relationship between numbers of missing calycoytes and numbers of missing gonads in embryos from eggs treated at the different ages (in minutes) indicated.

(v) *Effects of Age at Time of Treatment.*—To consider the effects of age at time of treatment it is necessary to describe briefly the basis for the age groupings used in Tables 1–3. The embryos analysed from the youngest group derive from eggs treated at 60, 62, 67, 70, or 75 min after laying respectively. This is before the time of migration of nuclei into the polar region and well before the formation of any pole cells (Rabinowitz 1941, Sonnenblick 1950). The treatments at these *early* pre-pole cell stages represent irradiations of the polar plasm alone.

In the second group (79–86 min) the treatments were given at 79, 83, 85, or 86 min respectively. Although nuclear movement into the polar region begins there is no pole cell formation during this period. Treatments at this *late* pre-pole cell stage also represent irradiations primarily of polar plasma rather than nuclei.

The third group (89–102 min) comprises embryos in which the movement of nuclei into the polar plasma has begun and there is active budding of pole cells. In these treatments, which were at 89, 91, or 102 min, many nuclei were in the region exposed to radiation.

In the next interval (103–119 min) pole cell formation is largely completed but pole cell mitoses continue. Most of the embryos in this group were close to 110 min of age when irradiated.

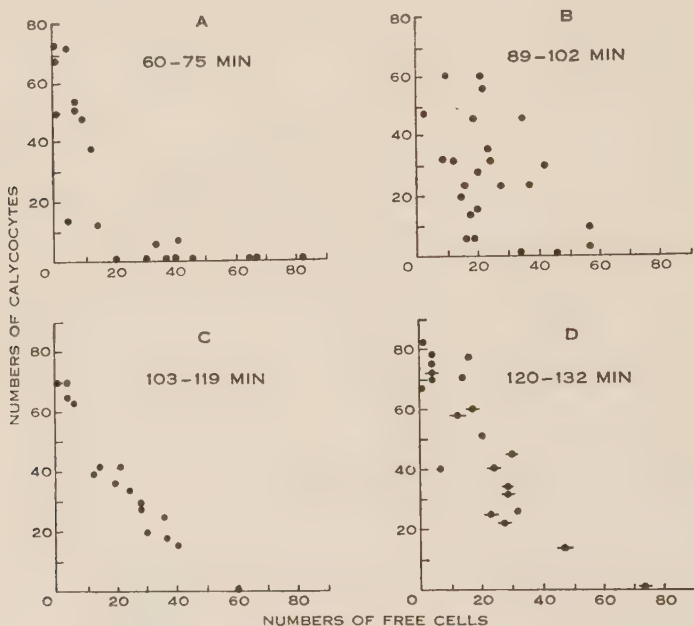


Fig. 4.—Relationship between numbers of calycoytes in the gut epithelium and the numbers of free cells in the lumen of the gut in embryos from the times of treatment indicated. These correspond to early pre-pole cell (A), late pre-pole cell (B), pole cell formation (C), and pole cell multiplication (D) stages respectively. —●— in D indicates the 124-min eggs which received a 4-min irradiation.

The older embryos are presented in two separate groups because some of them (124 min) were exposed to a double dose (4 min) compared to others (120 and 132 min) which received the 2-min dose. Pole cell mitoses continue in this period, near the end of which some of the inner pole cells begin the movement toward the underlying blastema layer. Shortly this becomes (156 min) a full-fledged migration to the interior of the egg.

From Tables 2 and 3 it is apparent that reduction of calycoytes is strong in the earlier periods, reaches its maximum during the period of pole cell formation

(89–102 min), and subsequently drops off markedly. For gonads the effects are seen to be least in the early period, but to reach their maximum simultaneously with the calycoocyte effects in the third period. They drop in the two later periods, but not as low as at the earliest time. From Table 3 which presents the proportions of embryos showing both calycoocyte and gonad effects, those showing only calycoocyte effects, those showing only gonad effects, and those unaffected in either way, the total calycoocyte effects and total gonad effects are readily obtained. The relationships of these are graphically presented in the summarizing figure (Fig. 6). The interpretation of these is dealt with in Section IV.

(vi) *Dosage Effects*.—Three different dosage levels were employed as represented by exposure times of 2, 3, and 4 min (Table 2). All the eggs of the first two groups were given 3-min exposures. The third group contained both 2- and 3-min exposures but, as no statistically significant difference was evident, they have been pooled.

TABLE 4  
EFFECTS OF ULTRAVIOLET DOSAGE ON CALYCOCYTES AND GONADS IN *D. MELANOGASTER*

Dose (min)	Age at Time of Treatment (min)	Number of Embryos Analysed	Mean No. Calycoocytes Missing	Mean No. Gonads Missing	Proportion Embryos Affected
2	120–132	14	13.3	0.72	0.29
4	124	11	34.7	1.37	0.91

Except for those given a 4-min exposure at 124 min all the remaining eggs received a 2-min treatment.

In terms of reduction in calycoocyte number (Table 4) the effect of doubling of the dose at 124 min as compared with 120–132 min is nearly triple. The same is true when proportions of affected embryos are considered. However, in the case of the gonads the mean number of missing gonads is almost exactly doubled with the double dose. Thus the effects of the higher dose are disproportionately greater on calycoocytes than on gonads at the only time for which adequate dosage data are available. An interpretation of this is offered in Section IV.

(vii) *Calycoocyte Number and Sex*.—Tables 2 and 5 show that calycoocyte numbers in the series of controls ranged between 62 and 83. It is evident from Table 5 that embryos with the lower numbers of calycoocytes are characterized by large gonads whereas those with higher number have small gonads. Gonad size has been the sole means of distinguishing sex in larvae of *D. melanogaster* (Kerkis 1931) and also in embryos (Poulson 1937; Sonnenblick 1941, 1950). Large gonads are characteristic of males, small gonads of females. Although the control series available here is not extensive, the correlation between calycoocyte numbers and gonad size is unequivocal. The embryo with 70 calycoocytes was damaged in the region of the



gonads and size could not be established with certainty. Although the sexual dimorphism with respect to calycocyte number is not likely to be of much practical use in separation of the sexes it is the only non-gonadal sex difference so far detected in embryos and early larvae. Its existence makes it clear that not all of the calycocyte reduction produced by the ultraviolet treatments is apparent.

(viii) *Large Cells of the Lower Middle Midgut*.—During the examination of the embryos of the experimental series it was observed that the region of the middle midgut below the calycocytes is characterized by cells much larger than cells of

TABLE 5  
CALYCYTES, "LARGE" CELLS OF MIDGUT, AND GERM CELLS IN *D. MELANOGASTER* CONTROLS  
AT 22 HR

No.	Condition	No. of Calycocytes	No. of "Large" Cells	No. of Cells Free in Gut Lumen	Gonads	
					Size	Av. No. of Germ Cells
1	Unhatched	62	15	0	Large	30
2	Unhatched	62	16	0	Very large	24
3	Unhatched	62	14	1	Large	14
4	Hatched	63	15	0	Large	20
5	Unhatched	66	14	0	Very large	20
6	Unhatched	70	16	0	—*	—
7	Unhatched	72	16	0	Small	8
8	Hatched	79	14	0	Small	10
9	Hatched	79	16	0	Small	10
10	Hatched	82	15	0	Small	16
11	Unhatched	82	16	0	Small	12
12	Unhatched	83	18	0	Small	—*

\* Damaged during fixation.

any region of the midgut possessing correspondingly large nuclei. It was at first thought that these large cells might have been derived from pole cells or other cells whose division had been inhibited by the ultraviolet treatment. Counts of the large cells demonstrated them to be a persistent feature and remarkably uniform in number not only in the treated embryos, but also in the controls where their numbers ranged from 14–18 (Table 5). A compilation of the data for embryos in which large cells as well as calycocytes were counted is given in Figure 5. This includes data from control as well as treated embryos. Reduction in number of large cells is seen to be relatively infrequent and not correlated with reduction in calycocyte numbers.

These large cells give rise to the very flat epithelial cells of the lower middle midgut described by Strasburger (1932) for the larva. They appear to be derived from the tip of the posterior midgut invagination and their constancy in the experimental materials is strong evidence for localization of the ultraviolet treatment to the pole cells.

(b) *D. melanogaster*—Other Effects

Effects other than those which have been dealt with above, but less susceptible to quantitative treatments were found. In all instances of severe reduction in calycoocyte number the midgut region failed to undergo the normal form change and remained sac-like (Plate 1, Fig. 4; Plate 2, Figs. 1–3). In those instances in which calycoocytes were recognizable in the epithelium they were contiguous (Plate 1, Fig. 4; Plate 2, Fig. 1). Moreover, with the exception of one embryo which suffered extensive posterior damage, the structure of the gut epithelium was completely continuous throughout, demonstrating successful union of anterior and posterior midgut rudiments. This was also true in those embryos which suffered some dorsal herniation. Herniation was infrequent among the early treated eggs (Table 2), although about 30–40 per cent. was found among those of the middle periods (89–102 and 103–119 min). None was present among later-treated embryos except for about 18 per cent. among those receiving the double dose at 124 min. Aside from the mechanical displacements arising from herniation and the gut and gonad disturbances these embryos were essentially normal.

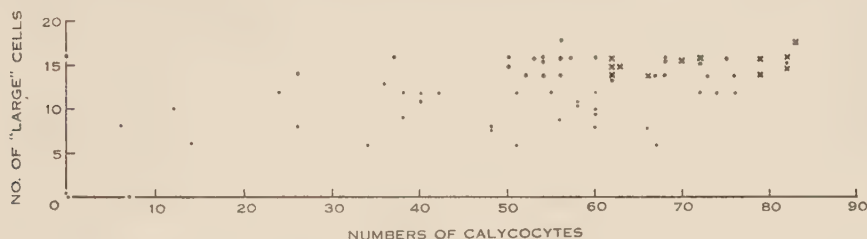


Fig. 5.—Relationship of "large" cell numbers to calycoocyte numbers in treated (●) and control (×) embryos.

A few cases of extensive posterior damage were found. In these it was clear that rather more than the pole cell region was exposed to the ultraviolet and damaged pole cells remained in a yolky mass at the posterior of the egg (Plate 2, Fig. 4). In the embryo figured the gut is incomplete and the peritrophic membrane is pushed out from above into the external yolk mass.

An interesting and unusual abnormality, found in a few embryos only, consisted of inverted malpighian tubules growing into the lumen of the gut (Plate 2, Fig. 5) instead of into the haemocoel. In the instance figured all four of the malpighian tubules can be followed. No instances of complete absence of malpighian tubules were encountered except in the few cases of extreme posterior damage.

(c) *L. cuprina*

The effects of the treatments of pole cells on the midgut of *L. cuprina* parallel very closely those described for *D. melanogaster*. The principal quantitative data are derived from counts of cuprophilic cells in the midguts of larvae which survived the treatment and, on being fed liver, attained the third instar. Cuprophilic cells were counted with the aid of the neotetrazolium procedure (Waterhouse and Stay 1955) by which they are readily distinguished from the adjacent lipophilic cells.

The data, summarized in Table 6, show a statistically significant reduction in cuprophilic cells in these surviving larvae. If the range in the controls is an expression of sex dimorphism then the effect may be rather greater than the table indicates. By itself this table is not very impressive, but taken together with data from sectioned material of unhatched larvae and embryos from the same and other series of treated eggs it is certainly quite significant. These showed conditions closely resembling those found in *D. melanogaster*. Characteristic features were the presence of considerable numbers of free cells in the gut lumen (Plate 4, Figs. 2, 3, and 4), the failure of the middle midgut to change from sac to tube (Plate 4, Figs. 3, 4, and 5), and the presence of free pole cells in the posterior yolk (Plate 4, Fig. 4) in more seriously damaged embryos.

TABLE 6  
L. CUPRINA: NUMBERS OF CUPROPHILIC CELLS IN THE MIDDLE MIDGUT  
OF THIRD-INSTAR LARVAE FOLLOWING ULTRAVIOLET TREATMENT IN THE  
EGG STAGE

Age (min)	Dose (min)	No. of Larvae	Mean No. of Cuprophilic Cells	Range
65-73	2	10	$137.6 \pm 5.5$	105-167
77-87	2	18	$137.1 \pm 7.1$	78-195
120	2	5	$118.0 \pm 7.7$	94-135
Controls	0	23	$152.7 \pm 5.5$	99-210

*L. cuprina* was most useful in determining the localization of the treatment in the polar region and in following the immediate effects of treatment. In one series of experiments eggs were treated during the period of pole cell budding and division, 71-79 min after oviposition, and embryos were fixed at intervals of 30, 60, and 90 min as well as at time of hatching of normals. The degree of localization of the ultraviolet in the polar region is strikingly clear in Plate 3, Figures 1, 2, and 3, in which the limits of the treated region are clearly demarcated.

The effects of the treatment of the pole cells are apparent in these same figures: cessation of mitosis and growth, vacuolization, clumping of chromatin, and some fragmentation of cytoplasm. At 1 hr following treatment there is in addition an increased basophilia of cytoplasm in the unfragmented pole cells (Plate 3, Fig. 4; Plate 4, Fig. 1) which enables them to be recognized more readily than in untreated controls.

Gonads were also strongly affected by the treatment. Although the data from sectioned material is not extensive enough for tabulation the following statements can be made about the effects on gonads. In the early-treated material (67-81 min, budding of pole cells, and mitosis) there were four gonads among 16 embryos, a mean of 0.25 per embryo, while among later-treated eggs (95-107 min, beginning

of blastoderm formation) there were 13 gonads in 14 embryos, a mean number of 0.93. In these instances the dosage was the same, 2 min. Control embryos fixed at the same times all contained two gonads each. In about half (three) of the controls the average number of germ cells per gonad was 24–28 whereas in the remainder (four) it was 13–16 per gonad. These counts were made before the time of onset of mitosis in later stages. Thus the sexual dimorphism in gonad size has its basis, as in *Drosophila*, in smaller or larger numbers of initial germ cells which enter the gonads.

In comparison with *D. melanogaster* the rate of embryonic development of *L. cuprina* under the conditions maintained during these experiments (25°C and 75–80 per cent. humidity) is considerably greater and larvae hatch is approximately 14 hr from oviposition. No pre-pole cell stages were treated in *L. cuprina* and no blastoderm stages were treated in *D. melanogaster*.

#### IV. DISCUSSION

The results described above, and summarized for *Drosophila* in Figure 6, are sufficiently clear to require little elaboration. However, they have an important bearing on problems relating to the determination and fate of the pole cells.

From the time that the term "pole cells" was applied to them by Weismann (1863) the significance of these cells aroused interest. Once Metschnikoff (1866) had traced them to the gonads in *Myastor* they provided the most striking and diagrammatic example of the separation of the germ line from the soma and were so employed by Weismann (1885) in his classical exposition. Later studies, primarily on lower Diptera, confirmed those of Metschnikoff and called attention to characteristic inclusions in the polar plasm (Ritter 1890; Kahle 1908; Hasper 1911), usually granular, which were always incorporated in the pole cells and the derived germ cells and which from then on were referred to as germ cell determinants (Hegner 1908, 1914). With the passing of the hey-day of organ-forming substances and particularly since the work of Huettner (1923) on *D. melanogaster*, in which the whole of the early work on pole cells and their relation to the gonads was reviewed, the term polar granules has been employed to refer to these inclusions. Still, the aura of determinism surrounding the particles has been persistent and will probably continue until their real function is better understood. The recent studies of Counce (1959) on several *Drosophila* species suggest an early breakthrough in this respect.

Huettner (1923) recognized, and his students Rabinowitz (1941) and Sonnenblick (1941, 1950) demonstrated that, despite the presence in them of polar granules, only a fraction of the pole cells in *D. melanogaster* reach the gonads. Both Huettner and Rabinowitz interpreted the early entry of a group of the pole cells to the interior of the egg just before blastoderm formation as the source of secondary yolk cells and considered the conspicuous group of pole cells which enter the posterior midgut invagination the source of the germ cells of the gonads. As some of these pole cells were observed in between cells of the midgut rudiment they were interpreted as passing through the gut wall in their migration to the gonads (Aboim 1945; Sonnenblick 1941, 1950) whereas others remained lost in the gut. The interpretation that, instead of being lost, the majority of pole cells become incorporated



as part of the midgut epithelium was put forward on the basis of descriptive embryology (Poulson 1947). Subsequently it appeared increasingly unlikely that passage

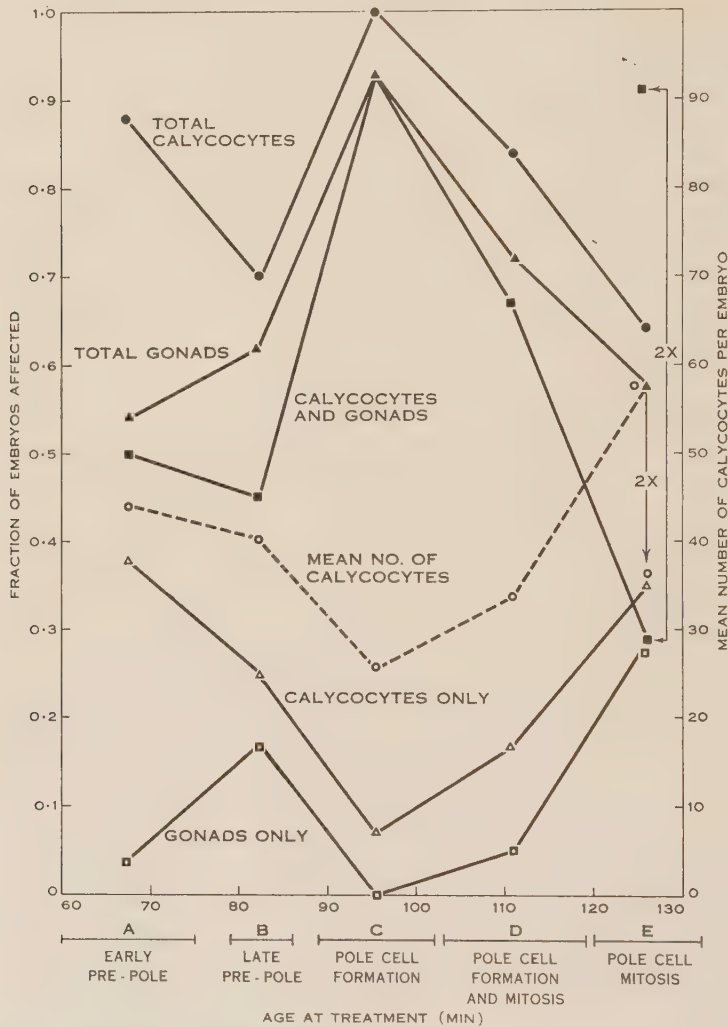


Fig. 6.—Graphic summary of effects of treatment at different ages.  $\square$  Embryos with gonads only affected;  $\triangle$  embryos with calycoytes only affected;  $\blacksquare$  embryos with calycoytes and gonads affected;  $\blacktriangle$  total gonad effects;  $\bullet$  total calycoyte effects. Broken line unites mean numbers of calycoytes per embryo for different times of treatment. Effects of the double dose on calycoyte number and on fraction of embryos affected is indicated at the right ( $2 \times$ ). *A*, the period 60–75 min is early pre-pole cell before entry of any nuclei into polar plasm; *B*, the period 79–86 min is pre-pole cell with nuclei beginning to enter polar plasm; *C*, the period 89–102 min is that of active pole cell formation; *D*, the period 103–119 min includes pole cell formation and mitosis; *E*, the period 120–132 min is chiefly one of pole cell mitosis.

of pole cells through the midgut represented the path to the gonads and much more probable that the early migration (pre-blastoderm) pole cells provided the source

of the germ cells of the gonads (Poulson 1950). Evidence from several lethal mutants in *D. melanogaster* strongly supports this view (Counce and Ede 1957; Poulson, unpublished data), as does a consideration of the evidence from the comparative embryology of Diptera.

In the lower forms (Nematocera) in which the pole cells have been traced directly to the gonads (*Chironomus* (Ritter 1890; Hasper 1911), *Miastor* (Metschnikoff 1866; Kahle 1908), *Sciara* (DuBois 1932; Butt 1934), *Simulium* (Gambrell 1933)) the total number of pole cells is small. These migrate to the interior before or during the blastoderm stage, and there is no association with the posterior midgut rudiment. Development of the egg is usually slow occupying several days or more.

Among the higher forms (Brachycera) which have been studied embryologically (*Calliphora* (Noack 1901; Pauli 1927), *L. sericata* (Fish 1947), *L. cuprina*, this paper, *Melophagus* (Lassman 1936), *Musca domestica* (Escherisch 1900; Reith 1925; Pauli 1927)) many pole cells are formed and there is an early inward migration similar to that observed in *Drosophila* as well as association of the majority of pole cells with the posterior midgut rudiment. Development in the egg is rapid and usually completed in less than 24 hr. An apparent exception to this may be *Phormia regina* in which according to Auten (1934) the early migrating pole cells go to the gonad and no pole cells appear to be associated with the posterior midgut. This case requires further investigation.

The only experimental evidence prior to that reported here which bears on the time of separation of the germ cells from the remaining pole cells is that of Geigy (1931). Geigy treated eggs of two different stages, "blastema" which corresponds to the later stages of pole cell formation and multiplication, and "blastoderm" at which time the first entry of pole cells is nearing completion. Of the 30 adults which he obtained from treatment in the blastema stage, 16 were wholly castrated (no gonads) and one partially castrated (one gonad), a mean of 0.9 gonads per fly. From treatment of blastoderm stage he obtained 13 adults of which five were partially castrated (one gonad) and none totally castrated (a mean of 1.6 gonads per fly). Although this difference might be attributed to shielding of some cells by others at a stage when they are more numerous, or as a drop in sensitivity to irradiation, it is most probably a reflection of the fact that a large fraction of the germinal pole cells have already passed to the interior of the egg. Moreover, mortality was very high in both stages, 0.63 at blastema and 0.91 at blastoderm, indicating either inadequate localization of treatment or persistent damage to cells other than germinal in their developmental fate. Since Geigy examined embryos only for immediate damage to pole cells and we did not attempt to raise any of our survivors to adulthood it is difficult to make closer comparison. The developmental study by Aboim (1945) gives no frequency data.

Our data (Fig. 6, total gonads) show that gonad effects are low when pre-pole cell stages are treated, that they rise as pole cell budding approaches, are at their maximum during budding, and decline in the later periods. Geigy's blastema stage probably corresponds to our 103-132-min groups, and his blastoderm stage is later than any used in our experiments with *Drosophila* although comparable to the later stages treated in *Lucilia*.

Unfortunately, the fact that our embryos and larvae were fixed at the time of hatching rather than before the onset of mitotic activity in the gonads between 16 and 18 hr makes it impossible for us to assess the gonad effects directly in terms of numbers of germ cells, except in a few extreme instances. Hence all the data have been expressed in terms of number of gonads per embryo. All the available data support the interpretation that the germ cells in *Drosophila* and in *Lucilia* are derived from the early migrating pole cells.

In the agonadic embryos very careful search for evidence of germ cells and empty gonad sheaths was made. While it is conceivable that such escaped attention, it is unlikely. In no instance of complete absence of germ cells were traces of gonad sheath found. In those exceptional cases where only two or three germ cells were present per gonad the sheath cells closely enveloped them. There was no evidence in any of these of a large sheath deficient in germ cells. It appears overwhelmingly likely that the sheath arises in response to inductive action of the germ cells which, in normal circumstances, reach the lateral mesoderm and aggregate. Other evidence for inductive action of germ cells was found by Counce and Selman (1955) in eggs subjected to sonic treatment and by Counce and Ede (1957) in the lethal mutant, *nas<sup>A</sup>* of *D. melanogaster*. Thus the agametic gonads found by Geigy (1931) and studied in detail by Aboim (1945) most probably arose through the action of less severely damaged pole cells reaching the embryonic mesoderm. Such cells might be fully capable of inductive action without capacity for further division. Evidences of damaged germ cells in gonads of treated embryos were found in *Lucilia* as well as in *Drosophila*. The inductive potency of dead or dying cells is a common phenomenon in embryogenesis.

Among the unsolved problems are: what determines which pole cells engage in early migration and how many do so in a given embryo? It may be that the first signs of sex differentiation are to be found in the numbers of early migrating pole cells at  $2\frac{1}{2}$  hr, about 7 hr before the appearance of the definitively ensheathed gonads at  $9\frac{1}{2}$ –10 hr as described by Sonnenblick (1941, 1950). It is suggestive that Rabinowitz (1941) reported variable numbers moving into the posterior midgut invagination. Seen in the light of our data on different modal numbers of calycoocytes in the two sexes these points are clearly worth further experimental study.

Correlation of deficiency of cuprophilic cells with damage to pole cells might be interpreted as a direct relation: pole cells becoming differentiated into cuprophilic cells; or as an indirect relation: pole cells inducing cuprophilic differentiation in other cells of the middle midgut. While each hypothesis has its merits, the former is better supported by our quantitative data for *Drosophila*, i.e. the inverse relation between calycoocyte and free-cell numbers, together with sex dimorphism in numbers of calycoocytes.

The high proportion of calycoocyte effects in *Drosophila* from treatments prior to the entry of nuclei into the polar plasm is suggestive of localization in the outer or more cortical polar plasm. The relatively low proportion of gonad effects from these early treatments indicates either recovery, avoidance by the entering nuclei of the more severely treated peripheral regions, or preferential formation of germinal cells. At the early time the polar granules form a somewhat dense (truncated



conical) arrangement (Counce 1959) which might provide effective shielding of the deeper polar plasm. These polar granules in *D. melanogaster* are rich in ribonucleic acids as has been shown by ribonuclease treatment (Poulson and Hilse, unpublished data) which removes them, along with other basophilic granules, from the cytoplasm. This is in agreement with the findings of Mulnard (1954) for the bruchid *Acanthoscelides oblectus* Say and the more recent reports of Bhuiyan and Shafiq (1959) on *Musca vicina* Macq. and Nicklas (1959) on *Miastor* sp. In the latter Nicklas found evidence for some protein as well as nucleic acid in the granules.

Since approximately 90 per cent. of the radiation from the source was of wavelength 2536 Å the principal absorbing materials were probably nucleic acids. Before the entry of nuclei to the polar plasm these must have been the cytoplasmic nucleic acids (ribonucleic acids) of the polar granules. However, it must not be forgotten that other materials (e.g. cell proteins) also absorb this wavelength. The shielding effect of the outer on the inner polar granules must become less effective as the granules become distributed among the initial pole cells during the process of budding and during the ensuing mitoses. Nuclear absorption becomes a factor from this time on and, with further mitoses and increasing numbers of pole cells, the inner cells are increasingly shielded by the more external pole cells. These points are of importance in interpreting the gonad and gut effects and the changes in their proportions at different times of treatment.

The correlation of gonad and calyccocyte effect is most nearly complete at the time of pole cell budding (89–102 min). All embryos with affected gonads have affected calyccocytes and the gonad and calyccocyte reductions in terms of mean numbers per embryo are greatest. Only a small fraction of embryos with affected calyccocytes showed no measurable gonad damage (Table 3, Fig. 6). In the subsequent periods there is a marked drop in all effects and an increased independence of gonad and calyccocyte effects. That this may be a consequence of increased shielding as well as of physical separation when the future germ cells begin their inward migration is suggested by the dosage effect (Tables 2 and 4; Fig. 6). Only by treatment at an even later stage when the first migration is complete would it be possible to distinguish between these.

The decrease in the degree of calyccocyte reduction from its maximum (at 89–102 min) to the minimum observed (120–132 min) appears to be a reflection of increasing pole cell numbers during this period. That pole cell mitoses are nearly completed by 132 min is indicated by the nearly linear inverse relation between calyccocyte and free cell numbers (Fig. 4D) at the two dosage levels employed.

The dosages employed require a brief comment. From the observations on *Lucilia* eggs at short intervals following treatment (Plate 3, Figs. 1–3) and from later stages of both *Lucilia* and *Drosophila* it is clear that the dosage levels used were such as to allow the majority of treated cells to remain intact. But for this the reciprocal relations between calyccocytes and free cells (Fig. 4) could not have been established.

It is of interest to compare these dosages with those which have been employed in experiments on mutagenesis in *D. melanogaster* (Altenburg, Altenburg, and Baker 1952; Muller *et al.* 1954). The dosage rate employed by the former was 100  $\mu$ W



sec/cm<sup>2</sup>/min. Such a dose for 8 min (800  $\mu$ W sec/cm<sup>2</sup>) was the lowest giving a sex-linked lethal mutation rate ( $1.4 \pm 0.5$  per cent.) readily detectable. Doubling the dose gave an increase to  $7.9 \pm 2.5$  per cent., but further increase in dose gave little further increase. Our basic treatment of 2 min represents a dose of about 1200  $\mu$ W sec/cm<sup>2</sup> while the double dose was about 2400  $\mu$ W sec/cm<sup>2</sup>. Thus one must conclude that dosages in the range used in experiments on mutagenesis result in a very high degree of damage to pole cells (and to other unshielded areas of the egg). The failure to obtain increased mutation rates at higher doses (Muller *et al.* 1954) is scarcely surprising.

Our data have another bearing on the mutagenic work on pole cells. This is the importance of the age of the embryo at which treatment is given. Clearly exposure at later ages will give lower rates, particularly if increased dosage is brought about by increasing exposure time rather than by increasing intensity, a fact previously appreciated by Muller *et al.* (1954) on other grounds. Since the "polar cap" stages employed in the mutagenic studies were no younger than 120–135 min after fertilization and our studies indicate that the germ cells are already beginning their inward migration during this period it is evident that in the later polar caps there will be relatively few germinal pole cells susceptible to radiation for they will be well within the egg. It is desirable therefore to add to the set of suggestions for future work on mutagenesis in pole cells (Muller *et al.* 1954) the recommendation that earlier stages (90–100 min), at which germinal pole cells are maximally exposed, be employed.

In our experiments the effects of doubling the dosage in the later period was much greater on the reduction of the calycoocytes than on the gonads. It appears from the essentially maximal effects of the 2-min dose for eggs of 89–102 min that this is a near optimal dose for experimental studies of the type undertaken here.

The bearing of these experiments on the genesis and structure of the larval middle midgut is considerable. The relationship between the non-germinal pole cells and the cuprophilic cells in *Lucilia* and the calycoocytes in *Drosophila* is clearly demonstrated. One of the puzzling differences between *Lucilia* and *Drosophila* has been the apparent absence in the latter of the striking mosaic arrangement of cuprophilic and lipid cells of the former, although Waterhouse and Stay (1955) reported that histochemical tests revealed that two cell types did, in fact, occur in *Drosophila* larvae. Another is the absence of the goblet cell type in *Lucilia* and related genera as compared with drosophilids. While the present studies cast very little light on the latter point they do clarify the former. A careful study of the preparations, control as well as experimental, shows that the calycoocytes of *Drosophila* embryos are interspersed among other cells with which they clearly alternate in certain of the lower sections of the middle midgut (Plate 1, Figs. 2 and 3). We refer to the other cell type as *interstitial* although the experimental evidence is all in favour of the insertion of the calycoocytes between the original cells of the posterior midgut rudiment at its junction with the anterior midgut rudiment. Evidences of failure to become incorporated into the epithelium are clearly present in Plate 2, Figures 1 and 3. Occasional free cells in the lumen show vacuoles suggestive of stages in the formation of the goblet of the calycoocyte. The mosaic arrangement

of calycoocytes and interstitial cells in the young larva bears a close resemblance then to the mosaic condition of the later larval stages of *Lucilia*. Since treated embryos of *Lucilia* show essentially the same condition of free cells and incompletely differentiated middle midgut (Plate 4, Figs. 2 and 3) the mosaic arrangement appears to originate in both as a consequence of the manner in which the pole cells enter the primitive midgut epithelium. As the larva of *Drosophila* grows, the calycoocytes, large to begin with, increase disproportionately to the interstitial cells which become relatively inconspicuous in the later instars so that the original appearance of a mosaic disappears.

Although the calycoocytes have been referred to here as goblet-like because of their shape, they appear to bear little resemblance to the rather differently shaped goblet cells of larval Lepidoptera (Waterhouse 1952). In fact, cells closely similar to calycoocytes have not been reported outside the Drosophilidae.

The presence in the embryonic midgut of *Drosophila* of a previously unrecognized cell type, the "large" cell is clearly demonstrated in this material. These cells, characteristic of the lower middle midgut below the calycoocytes, remained remarkably constant in number in the treated as well as in the controls (Fig. 5), indicating that the cell group from which they take their origin was rarely subject to the radiation employed in these experiments and that their origin in the embryo is quite independent of the calycoocytes. In the later larva these become the very large flat cells of the lower middle midgut epithelium described by Strasburger (1932).

In a few of the embryos of *Drosophila* the presence of "inverted" malpighian tubes was encountered (Plate 2, Fig. 5). All four tubules were found to be directed into the lumen of the midgut, demonstrating that morphogenesis of these organs can proceed even in such an abnormal spatial orientation. No case of missing malpighian tubules or other evidence of direct radiation damage to malpighian tubules was found. These observations and the comparative rarity of other abnormalities (except in the cases of extreme posterior damage) indicate a high degree of localization of the radiation treatment in these experiments. It is probable that much of the herniation encountered was non-specific in origin as Goldman and Setlow (1956) observed in their work on whole egg irradiation.

The failure of larvae with greatly reduced numbers of cuprophilic cells (*Lucilia*) or calycoocytes (*Drosophila*) to hatch or to grow and metamorphose (most die within 24 hr of hatching if they hatch at all) suggests that these cells are essential to larval life in these two organisms. Further investigations of their structure and functions are clearly desirable.

In other animal groups relationships between endodermal rudiments and the primordial germ cells have been the subject of considerable study and speculation. In the vertebrates in particular a good deal of controversy has revolved around the question of the origin of the germ cells. The subject has been reviewed by Nieuwkoop (1949) and Burns (1955). The evidence indicates an intimate relationship between the earliest germ cells and endoderm. Recently Mintz (1959) has traced the germ cells of the mouse step by step from the yolk sac endoderm into the mesoderm where the definitive gonad is differentiated. The parallel to the situation in *Drosophila*

and *Lucilia* is thus remarkably close. While it is far from clear, either in vertebrates or insects, precisely what factors operate in deciding whether a given cell becomes a germ cell or remains as part of the endoderm, further studies on insect material offer considerable promise for the solution of this basic problem.

## V. ACKNOWLEDGMENTS

The authors wish to express their thanks to Mrs. Alice Halsey for valuable assistance with certain of the materials, to Mr. L. Marshall and Mr. D. Wilson for their aid in the preparation of the illustrations, and to Mr. W. Rafferty for construction of the apparatus used in the irradiations.

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## EXPLANATION OF PLATES 1–4

### PLATES 1 AND 2

All figures are of *D. melanogaster* embryos fixed in formol–alcohol–acetic acid at 24 hr after laying, sectioned at 7  $\mu$ , and stained with Harris's haematoxylin. The magnification for all figures is in Figure 2 for Plate 1 and in Figure 4 for Plate 2. Embryos in Plate 1, Figures 1, 3, and 4, and Plate 2, Figures 1, 2, and 4, were irradiated for 2 min, in Plate 1, Figure 2, for 3 min, and in Plate 1, Figure 4, for 4 min. In Plate 1, Figures 1, 2, and 3 show no effects of treatment and are comparable with control embryos. *C*, calycocytes; *F*, cells free in lumen; *I*, interstitial cells; *IMT*, involuted malpighian tubules; *L*, "large" cells; *MMG*, middle midgut epithelium; *PM*, peritrophic membrane; *YF*, free cell in yolk herniated from gut

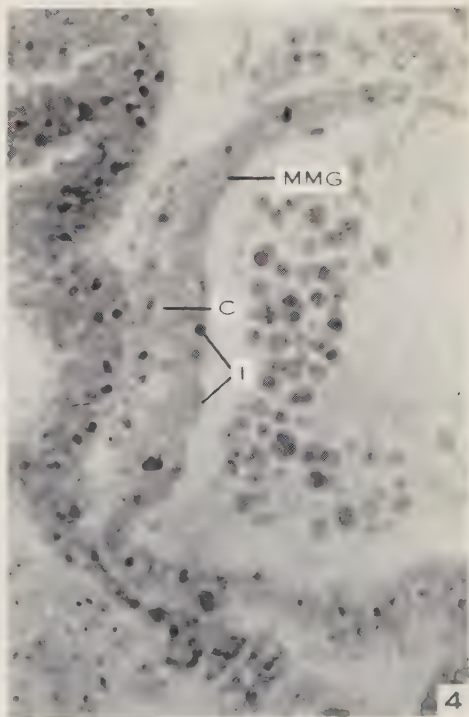
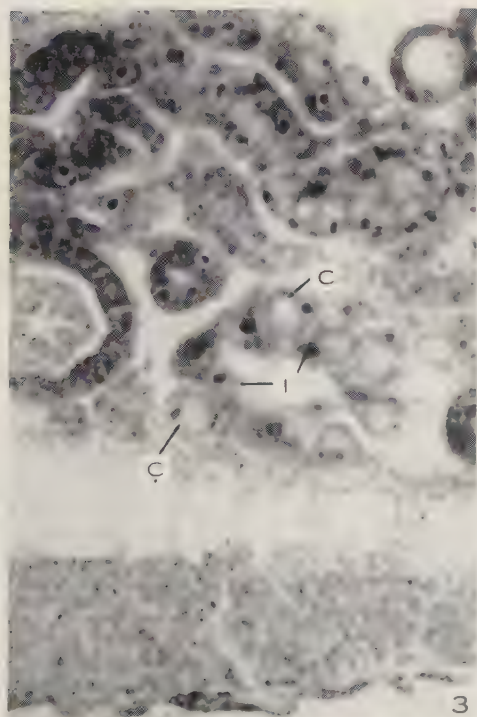
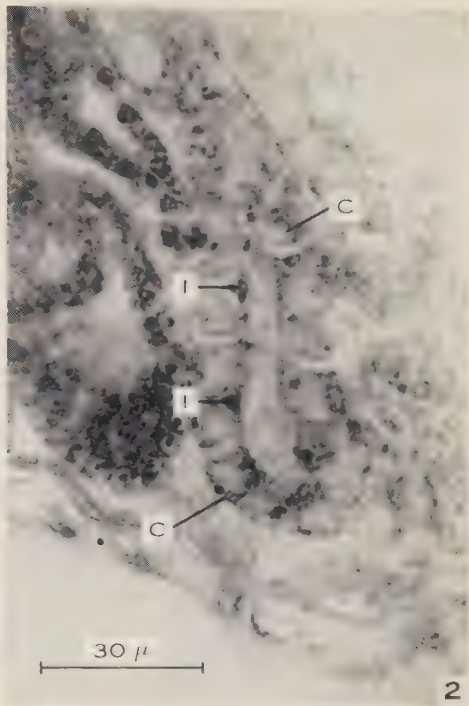
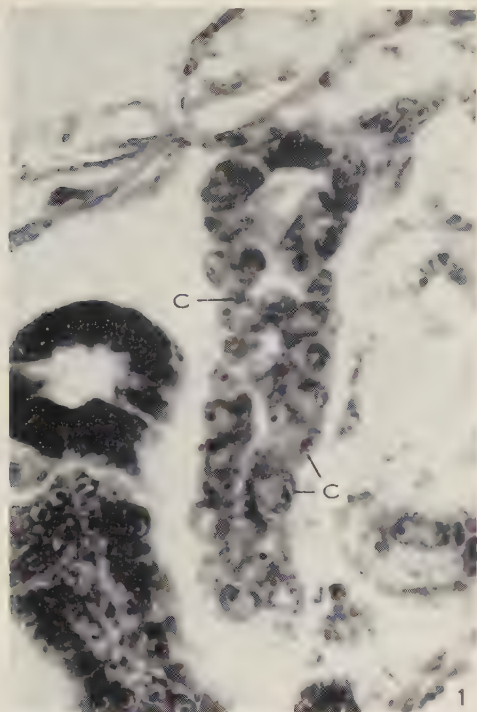
### PLATE 1

- Fig. 1.—Longitudinal section. Calycocyte region in embryo with 67 calycocytes. Irradiated 132 min after laying.
- Fig. 2.—Longitudinal section showing calycocytes and interstitial cells. Irradiated 67–70 min after laying.
- Fig. 3.—Transverse section of posterior calycocyte region showing calycocytes and interstitial cells. Irradiated 120 min after laying.
- Fig. 4.—Calycocytes and interstitial cells in embryo irradiated at 102 min developmental age.

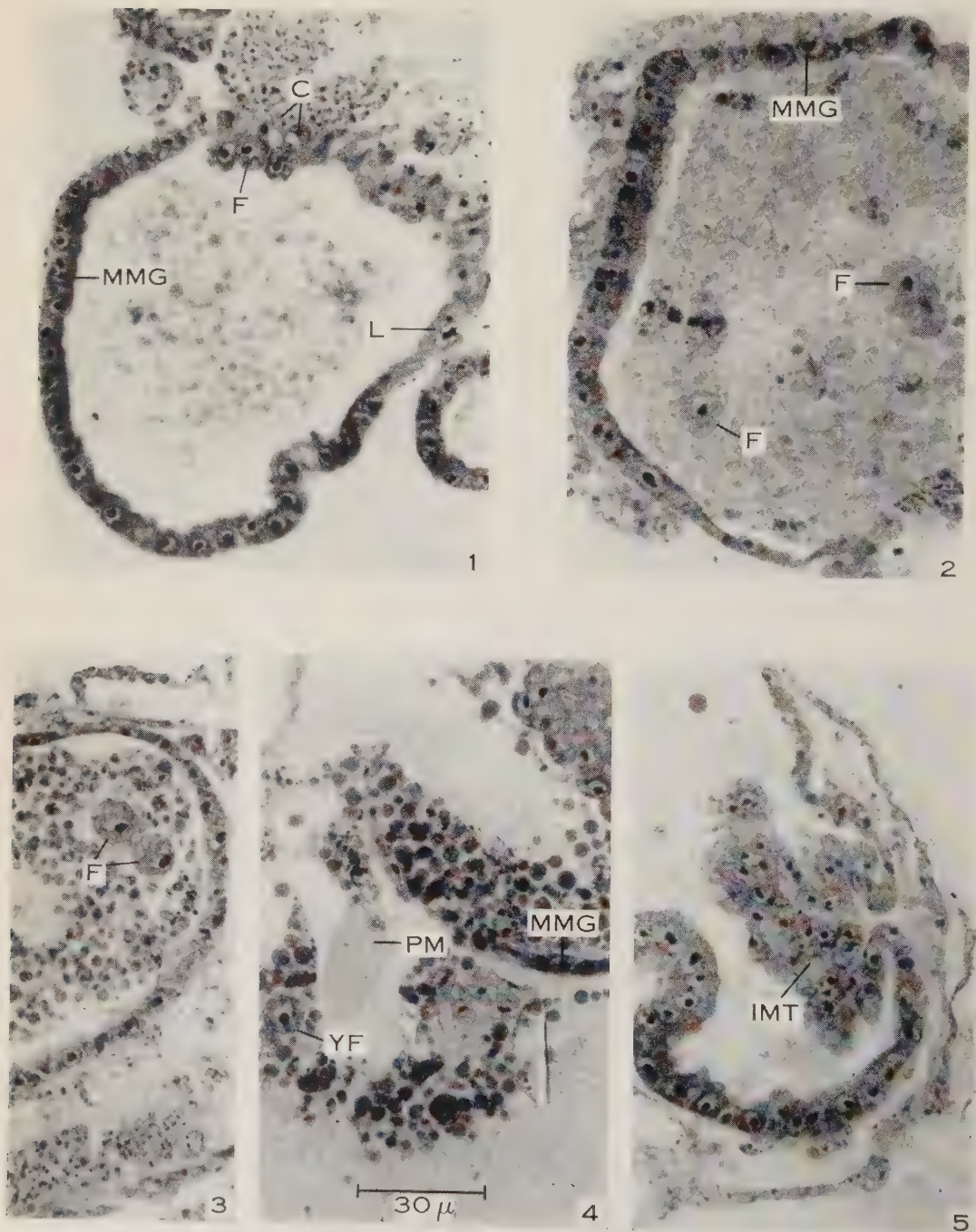
### PLATE 2

- Fig. 1.—Free cells in gut lumen. This embryo had 22 free cells and 56 calycocytes and had been irradiated at 102 min developmental age.
- Fig. 2.—Free cells in yolk in embryo with few (less than 20) calycocytes. Irradiated at 90 min after oviposition.
- Fig. 3.—Free cells and partially digested yolk in gut lumen. Irradiated 124 min after laying.
- Fig. 4.—Free cell in yolk herniated from gut. The peritrophic membrane is also visible. Irradiated 102 min after laying.
- Fig. 5.—Invaginated malpighian tubules from embryo irradiated for 2 min at 102 min developmental age.

POLE CELLS AND MIDGUT DIFFERENTIATION IN DIPTERA

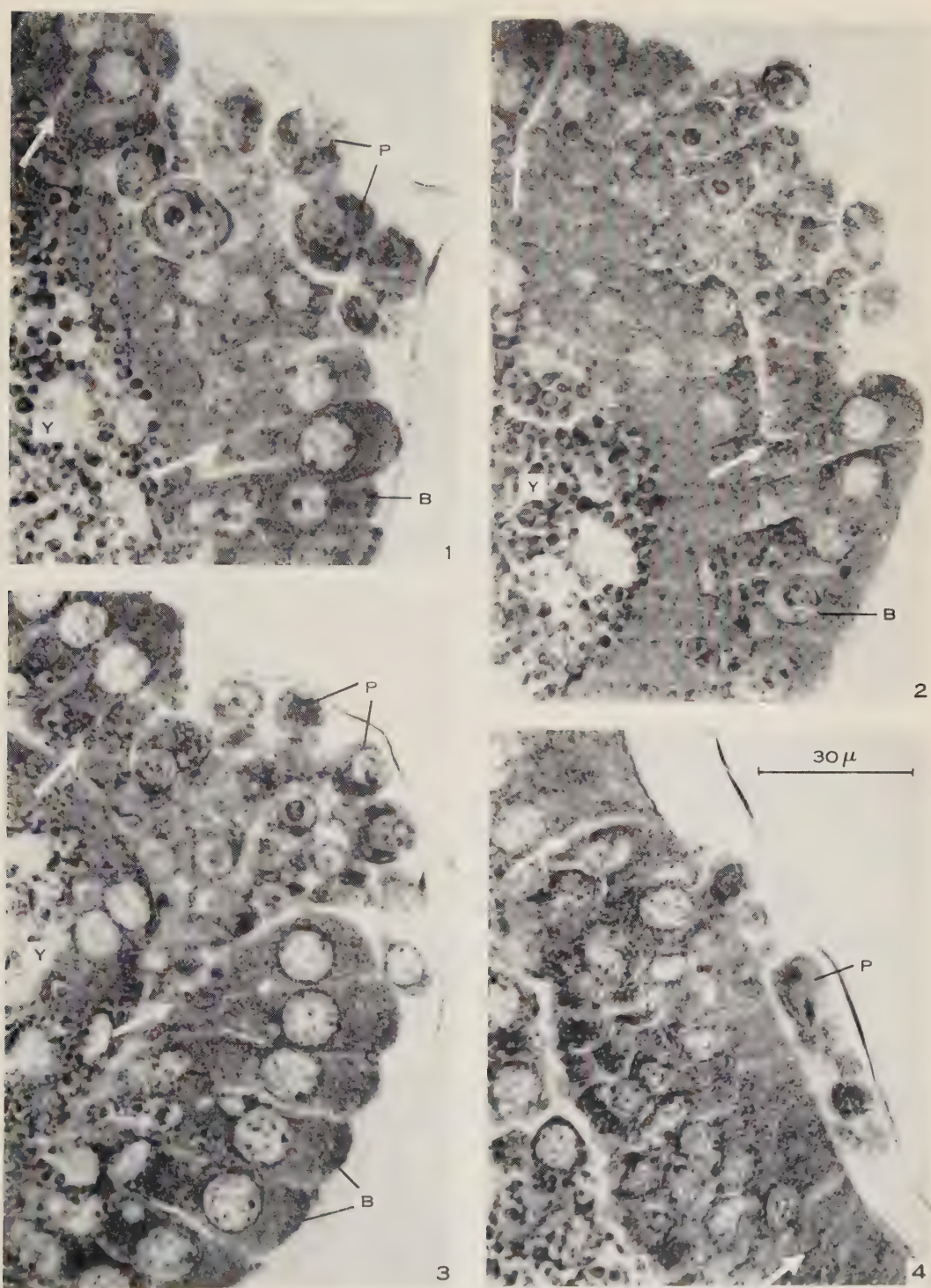


POLE CELLS AND MIDGUT DIFFERENTIATION IN DIPTERA



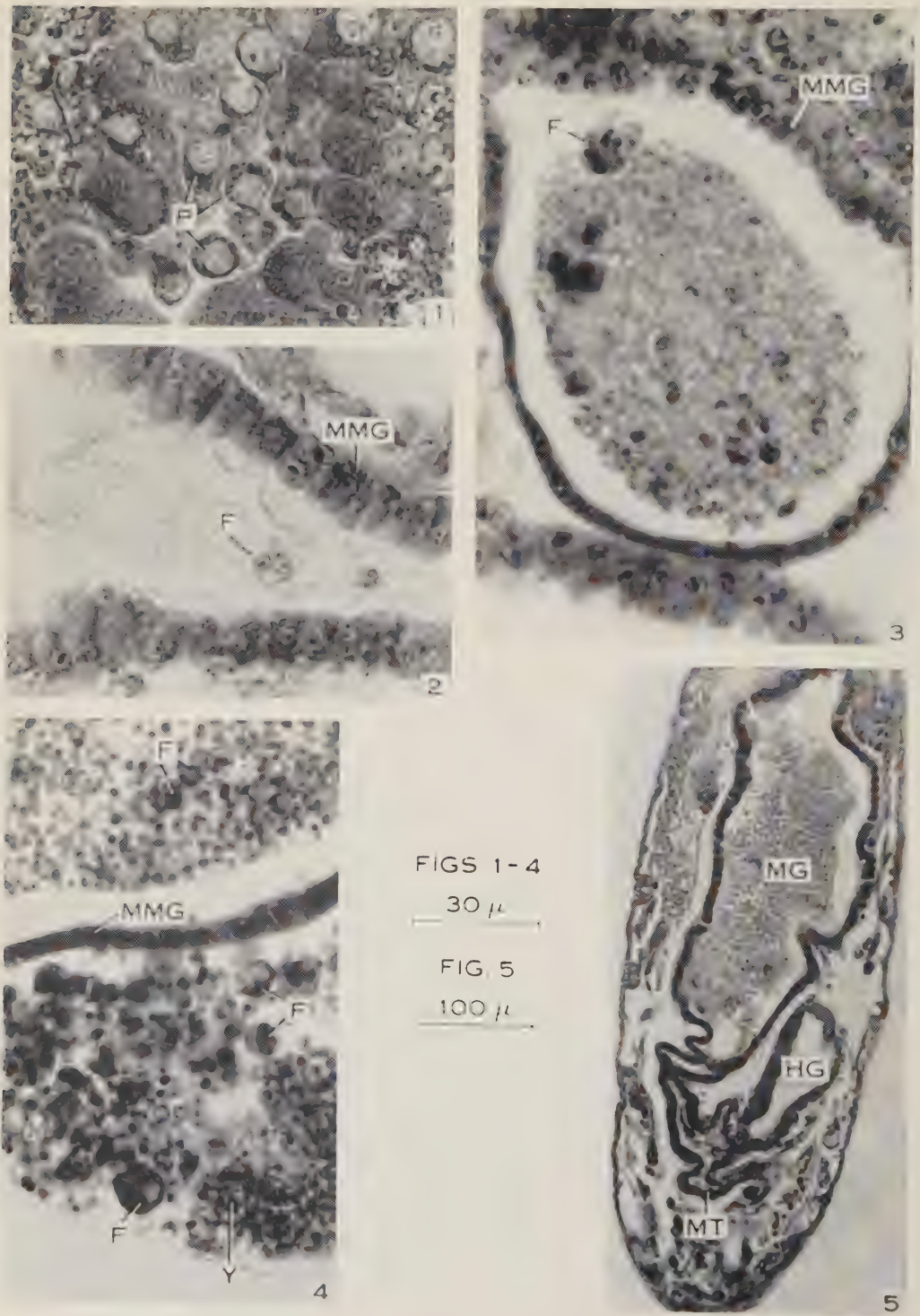


POLE CELLS AND MIDGUT DIFFERENTIATION IN DIPTERA





POLE CELLS AND MIDGUT DIFFERENTIATION IN DIPTERA



## PLATES 3 AND 4

All figures are of *L. cuprina* embryos fixed in formol-alcohol-acetic acid, sectioned at  $9\ \mu$ , and stained with Harris's haematoxylin. Embryos in Plate 3, Figures 1-4, and Plate 4, Figures 1, 2, and 4 were irradiated for 2 min, in Plate 4, Figures 3 and 5, for 4 min. Scale of magnification for Plate 3 given in Figure 4. *B*, blastoderm; *F*, cells free in lumen; *HG*, hindgut; *MG*, midgut; *MMG*, middle midgut epithelium; *MT*, malpighian tubules; *P*, pole cells; *Y*, yolk

## PLATE 3

- Figs. 1-3.—Longitudinal sections showing limited, but progressively smaller zones of ultra-violet damage (indicated by arrows) at the posterior poles of three eggs. Figures 1 and 3 irradiated at 71-73 min and Figure 2 at 77-79 min after laying. Fixed at 92 min.
- Fig. 4.—Treated pole cells near posterior pole of egg. Irradiated 71-73 min after laying. Fixed at 132 min.

## PLATE 4

- Fig. 1.—Pole cells in posterior midgut invagination. Irradiated 71-73 min after laying. Fixed at 132 min.
- Fig. 2.—Free cells in gut lumen. Irradiated 65-67 min after laying. Fixed at 24 hr.
- Fig. 3.—Free cells in gut lumen. Irradiated 73-77 min after laying. Fixed at 24 hr.
- Fig. 4.—Free cells and partially digested yolk in gut lumen (above). Posteriorly herniated material containing free cells can be seen in the lower half of the photograph. Irradiated 65-67 min after laying. Fixed at 24 hr.
- Fig. 5.—Horizontal section of embryo fixed at 24 hr, showing general details of midgut, hindgut, and entry of malpighian tubules. Irradiated 65-69 min after laying.

## STUDIES ON CHITIN

### IV. THE OCCURRENCE OF COMPLEXES IN WHICH CHITIN AND PROTEIN ARE COVALENTLY LINKED

By R. H. HACKMAN\*

[Manuscript received June 29, 1960]

#### *Summary*

Samples of chitin have been prepared from the cuticles of insects and crustacea, from cuttlefish shell, and from the skeletal pen of squid. In every case protein was bound by covalent bonds to the chitin so forming stable complexes (glycoproteins). Glycoproteins from different sources contained differing amounts of chitin and protein, the ratio of chitin to protein varying from 1 : 1 to 20 : 1. The protein would appear to be linked to the chitin through aspartyl or histidyl residues or both and each glycoprotein is polydisperse. The chitin preparations included glycoproteins which contained  $\alpha$ - and  $\beta$ -type chitins and also a third and different type of chitin. The amino acid composition of the protein components of the glycoproteins has been determined. It is probable that chitin does not occur uncombined with protein.

#### I. INTRODUCTION

Chitin occurs in the cuticles of all arthropods and in certain other invertebrates, e.g. squid and cuttlefish. It is always associated with protein and, for example, in crustacea the cuticle may also be heavily calcified. For reviews on the distribution of chitin in nature see Richards (1951, 1958) and Rudall (1955). Chitin, which was originally defined as the cuticular material insoluble in hot aqueous alkali, is a polymer composed of 2-acetamido-2-deoxy- $\alpha$ -D-glycopyranose (i.e. *N*-acetyl-D-glucosamine) residues linked in the 1,4- $\beta$ -glucosidic manner of cellulose. Its molecular weight is not known with any degree of accuracy and estimates range from a chain composed of from several hundred to one thousand residues.

Chitin is usually prepared from cuticles by repeatedly extracting them with hot dilute alkali (e.g. 1*N* aqueous sodium hydroxide at 100°C). If necessary the cuticles are decalcified prior to treatment with alkali. Such drastic treatment may well bring about structural changes in the chitin, in particular removal of pendent groups (e.g. proteins, peptides, or acetyl groups) and fragmentation. The presence of pendent groups may be important biologically and so it is of interest to know if such groups are present and if so the manner in which they are linked to the chitin chain.

The chitin which occurs in arthropods and also in some other invertebrates is designated  $\alpha$ -chitin to distinguish it from the  $\beta$ -chitin which occurs in some parts of certain invertebrates, e.g. the skeletal pen of the squid and the chaetae of annelids. There is no record of  $\beta$ -chitin occurring in arthropods.  $\beta$ -chitin, which is distinguished from  $\alpha$ -chitin by its X-ray diffraction pattern, was first described by

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Lotmar and Picken (1950). Work on  $\beta$ -chitin has been continued by Rudall (1955) who concluded that the  $\beta$ -chitin structure appears to be associated with collagen-type cuticles, whereas the  $\alpha$ -chitin structure replaces collagen-type cuticles.  $\beta$ -chitin is converted to  $\alpha$ -chitin by the action of cold 6N hydrochloric acid (Rudall, personal communication).

For more than one hundred years there has been speculation in the literature concerning the interaction between components of the insect cuticle. The old idea that the insect cuticle is a chitinous matrix impregnated with other materials has gradually given way to a consideration of the existence in the cuticle of a glycoprotein—a complex formed from chitin and protein, the two major components of the cuticle. Hackman (1955) and Hackman and Goldberg (1958) have shown that there is a weak bonding between chitin and some of the proteins in the insect cuticle, while in the larval cuticle of *Agrianome spinicollis* McL. much of the protein is bound to chitin in the form of a glycoprotein. Foster and Hackman (1957) have shown that in the cuticle of another arthropod, the crab *Cancer pagurus* L., part of the protein is bound chemically to chitin and the glycoprotein so formed is polydisperse.

In this paper a study has been made of the chitin-containing glycoproteins which occur in the cuticle of insects and crustacea, in the skeleton of cuttlefish, and in the skeletal pen of squid. Glycoproteins containing  $\alpha$ - and  $\beta$ -type chitins have been investigated.

## II. EXPERIMENTAL AND RESULTS

### (a) *Lucilia cuprina* (Wied.)

Chitin was prepared from washed empty puparia of *L. cuprina* by extracting the powdered material with 1N aqueous sodium hydroxide at 100°C until no further colour was extracted. The residue was collected by centrifugation, washed once with water, and dialysed against running tap water for several days until the pH of the supernatant was no longer alkaline. The chitin was then dialysed against distilled water for 24 hr. changing the distilled water several times during this period, collected by centrifugation, washed three times with ethanol, three times with ether, and finally dried *in vacuo* over phosphorus pentoxide. Yield 36.6 per cent. (Found: N, 7.0%; ash, nil. Calc. for  $(C_8H_{13}O_5N)_x$ : N, 6.9%).

A sample of this chitin (20 mg) was hydrolysed by heating for 17 hr with 5.7N aqueous hydrochloric acid (20 ml) in a sealed evacuated tube at 105°C. The hydrolysate was evaporated to dryness *in vacuo* at 30°C, dried *in vacuo* over solid potassium hydroxide, and the residue dissolved in 10 per cent. isopropanol (1 ml). The solution was subjected to ionophoresis on paper (Whatman No. 3 filter paper, acetate buffer pH 5 and ionic strength 0.19, potential 20 V/cm for the effective length of 50 cm for 60 min (Foster 1952, 1957)). Three spots were detected with ninhydrin, one intense spot (glucosamine) and two weak spots (one acidic and one basic amino acid). The amino acids, by comparison with the behaviour under similar conditions of known amino acids were identified as aspartic acid and histidine. Glucosamine was identified by the position it occupied on the paper and by its reaction with the Elson and Morgan reagent.



To confirm the presence of aspartic acid and histidine the ionophoresis on paper was repeated using an acetate buffer of pH 3·6, a phosphate buffer of pH 7·5, and carbonate buffers of pH 9·7 and 10·7. All the buffers had an ionic strength of 0·2. In these experiments aspartic acid, glutamic acid, lysine, arginine, histidine and glucosamine were included as controls. At pH 3·6 glutamic acid, at pH 7·5 histidine, at pH 9·7 lysine, and at pH 10·7 arginine remained at the origin. At all these pH values the spot corresponding to the acidic amino acid behaved in a manner identical with that of authentic aspartic acid and the basic amino acid in a manner identical with that of authentic histidine. At none of the pH values did any additional spots appear.

Further confirmation of the identity of the amino acids was obtained by filter paper partition chromatography. The hydrolysate was subjected to ionophoresis on paper, the position of the two amino acids determined by the use of guide strips, and the areas of paper containing the amino acids cut out. These pieces of paper were either woven into a strip of Whatman No. 1 filter paper (Boggs 1952) or the amino acids eluted and spotted on to a strip of Whatman No. 1 filter paper. The paper strips were subjected to chromatography using the solvent systems and spray reagents described by Hackman and Lazarus (1956) together with the additional solvent system methanol-water-pyridine (Smith 1958). Appropriate amino acids were used as controls on all paper strips and the two amino acids were identified as aspartic acid and histidine.

#### (b) *Agrianome spinicollis*

(i) Larval cuticles were prepared as described by Hackman and Goldberg (1958) and subjected to digestion with papain. Experiments showed that papain removed more protein from the cuticles than did either pepsin or trypsin. Finely powdered cuticle (100 mg) was digested at 70°C with papain (10 mg) in phosphate buffer (25 ml, Sørensen 1909) containing 0·01M sodium sulphide. At 2-hourly intervals a further 10 mg papain and 30 mg sodium sulphide were added and the total time of digestion was 6 hr. Soluble nitrogen was determined after 2, 4, and 6 hr. After 2 hr no more protein was digested and the nitrogen content of the solution corresponded to a residue containing 95 per cent. chitin and 5 per cent. protein. The residue was washed thoroughly with water, alcohol, and ether and dried *in vacuo* over phosphorus pentoxide. The absence of sulphur-containing amino acids in this residue established the absence of papain (*A. spinicollis* larval cuticles after acid hydrolysis do not give any cystine, cysteine, or methionine). The cuticular residue was hydrolysed with hydrochloric acid as described in Section II(a), the amino acids present in the hydrolysate identified (filter paper partition chromatography), and their concentrations estimated by visual comparison of the intensities of the coloured spots with those given by standard solutions of the amino acids. The results expressed as mg amino acid obtained from 1 g protein (associated with the chitin) are given in Table 1.

(ii) Chitin was prepared from the larval cuticles of *A. spinicollis* by the method described above for *L. cuprina* puparia. When subjected to hydrolysis with hydrochloric acid the degradation products were identified as glucosamine, aspartic acid,

and histidine by ionophoresis and chromatography on paper as described in Section II(a). The aspartic acid and histidine were present only in small amounts. Because of the unknown amount of decomposition which occurred during hydrolysis it was not possible to determine accurately the concentration of the aspartic acid and histidine present in the sample of chitin. However, an estimate of the amount of each present in the hydrolysate was obtained by visual comparison of the intensities

TABLE 1  
AMINO ACID COMPOSITION OF PROTEIN COMPONENTS OF GLYCOPROTEINS  
Amino acid composition expressed as mg amino acid obtained from 1 g protein

Amino Acid	Source of Glycoprotein			
	Larval Cuticle of <i>Agrianome spinicollis</i> (papain)	Cuticle of <i>Scylla serrata</i> (EDTA)	Cuttlefish Shell (EDTA)	<i>Loligo</i> Skeletal Pen (lithium thiocyanate)
Alanine	84	90	95	121
Arginine	82	190	47	24
Aspartic acid	125	109	142	55
Cystine and/or cysteine	Absent	Absent	43	20
Glutamic acid	139	97	52	42
Glycine	Trace	Trace	80	102
Histidine	37	51	55	43
Hydroxyproline	Absent	Absent	Absent	Absent
Leucine and/or isoleucine	103	72	47	89
Lysine	Trace	Trace	39	20
Methionine	Absent	Absent	Absent	Absent
Phenylalanine	78	109	35	28
Proline	55	38	41	58
Serine	Trace	Trace	38	29
Threonine	113	78	26	33
Tyrosine	171	120	129	62
Valine	138	129	63	56

of the coloured spots with those given by standard solutions of the amino acids. There were two histidine and one aspartic acid residue for each 400 glucosamine residues.

(c) *Scylla serrata* (Forskål)

(i) Chitin was prepared from the finely powdered, hair-free carapace of the crab *S. serrata* by extraction with aqueous ethylenediaminetetra-acetic acid (EDTA) at pH 9 and pH 3 as outlined by Foster and Hackman (1957).

Uncooked shell from recently caught crabs was cleaned of all flesh, air dried, and ground to a powder in a vibrating ball mill. Powdered shell (50 g) was added to a solution of EDTA (235 g) in water (1.5 l.) which had been adjusted to pH 9. The solution was shaken gently overnight, the insoluble material collected, and washed three times with very dilute ammonia (pH 9) and three times with water.

The residue was shaken gently overnight with a solution of EDTA (2.5 g) in water (500 ml) which had been adjusted to pH 3. The insoluble material was washed three times with water, three times with dilute ammonia (pH 9), and three times with water again. Pigment was removed by extracting the residue repeatedly with ethanol, and free lipid by extraction with ether. Yield 7.1 g (14.2 per cent.). (Found: N, 8.3% (on ash-free basis) and ash (550°C) 0.43%). A sample of this chitin was hydrolysed with hydrochloric acid and the amino acids in the hydrolysate identified and their concentrations estimated as described in Sections II(a) and II(b). The results are given in Table 1.

(ii) A second sample of this chitin was heated with 1N aqueous sodium hydroxide at 100°C for 60 hr changing the alkali five times during this period. The residue (Found: N, 6.9%) was isolated and hydrolysed as described in Section II(a). The 10 per cent. *isopropanol* extract was subjected to ionophoresis and chromatography on paper. Glucosamine and small amounts of aspartic acid and histidine were identified, no other compounds were detected.

#### (d) *Cuttlefish*

(i) Cuttlefish shell, collected from beaches on the south coast of New South Wales, was ground to a fine powder in a vibrating ball mill. The finely powdered shell was decalcified with aqueous EDTA at pH 9 and pH 3 as described in Section II(c) for powdered crab shell. The yield of chitin was 4.4 per cent. (Found: N, 11.6% (on ash-free basis) and ash (550°C) 5.2%). A sample of this chitin was hydrolysed with hydrochloric acid and the amino acids in the hydrolysate identified and their concentrations estimated as described in Sections II(a) and II(b). The results are given in Table 1.

(ii) A second sample of cuttlefish chitin was heated with 1N aqueous sodium hydroxide at 100°C for 60 hr, changing the alkali three times during this period. The residue was isolated (Found: N, 7.1%), hydrolysed with hydrochloric acid, and the 10 per cent. *isopropanol* extract subjected to ionophoresis and chromatography on paper. Only glucosamine, aspartic acid, and histidine were detected and the concentrations of aspartic acid and histidine were noticeably less than those obtained from *L. cuprina* or *S. serrata* chitins prepared in a similar manner.

#### (e) *Squid Skeletal Pen (Loligo sp.)*

(i) The skeletal pen contained 12.7 per cent. nitrogen and left no residue on heating at 550°C. A sample of the pen was hydrolysed with hydrochloric acid and the amino acids in the hydrolysate identified. The amino acid composition of the protein present in the intact pen was qualitatively similar to that of the protein in the glycoprotein prepared by the method described in Section II(e)(ii). Quantitatively the only marked difference was in the tyrosine content. The protein of the intact pen contained about twice as much tyrosine as the protein of the glycoprotein.

(ii) Powdered skeletal pen (250 mg) and aqueous lithium thiocyanate (50 ml, saturated at room temperature) were heated at 130°C for 2 hr with intermittent

shaking. A clear colloidal syrupy solution formed which was poured into 50 per cent. aqueous acetone (150 ml) and allowed to stand overnight. The precipitate was collected by centrifugation, washed with water until free from lithium thiocyanate, then with ethanol and ether and dried *in vacuo* over phosphorus pentoxide. Yield 102 mg. (Found: N, 8.1%; ash, nil). A sample of this chitin was hydrolysed with hydrochloric acid and the amino acids in the hydrolysate identified and their concentrations estimated. The results are given in Table 1.

(iii) The skeletal pen (500 mg) was heated with 1N aqueous sodium hydroxide (25 ml) at 100°C for 5 days, changing the alkali each day. The residue was isolated as described in Section II(a). (Found: N, 6.8%; ash nil). Acidic hydrolysis (HCl) of the residue gave only glucosamine, aspartic acid, and histidine.

#### (f) Other Chitin Preparations

Chitins prepared by the alkaline digestion of the decalcified shell of the marine crayfish *Jasus verreauxi* H. M. Edw., of the elytra of adult *Aphodius howitti* Hope, and of the decalcified shell of *Spirula spirula* L. were subjected to acidic hydrolysis and the hydrolysates examined by ionophoresis on paper. Only glucosamine, aspartic acid, and histidine were detected.

#### (g) Extraction with Aqueous Phenol

An attempt was made to extract protein from chitins prepared as described in Sections II(b)(i), II(c)(i), II(d)(i), and II(e)(ii).

Chitin (500 mg) was extracted with 90 per cent. aqueous phenol (20 ml) for 24 hr at room temperature in an atmosphere of nitrogen. The chitin was collected by centrifugation and re-extracted twice more with 20-ml portions of aqueous phenol, the duration of each extraction being 24 hr. The residue was thoroughly washed with methanol and ether and dried *in vacuo* over phosphorus pentoxide. Recovery was almost theoretical and the amino acid content of the residue was not materially altered from that of the chitin before extraction with phenol.

#### (h) Solution in Aqueous Lithium Thiocyanate

The four samples of chitin (Sections II(b)(i), II(c)(i), II(d)(i), and II(e)(ii)) were dispersed in aqueous lithium thiocyanate, saturated at room temperature as described in Section II(e)(ii). A syrupy colloidal solution formed within 1 hr. Any insoluble material was removed by centrifugation and the solution fractionally precipitated with acetone (see Foster and Hackman 1957) to give a series of chitin fractions. Each fraction was collected by centrifugation, washed with water until free from lithium thiocyanate, then with acetone, and dried. The fractions were hydrolysed with hydrochloric acid and the amino acids in the hydrolysates identified. In no case was a protein-free chitin fraction obtained and the amino acid composition of each fraction appeared to be similar to that of the chitin preparation from which it was prepared.



## III. DISCUSSION

Samples of chitin which have been prepared by alkaline digestion of insect cuticles (puparia of *Lucilia cuprina*, larvae of *Agrianome spinicollis*, and elytra of *Aphodius howitti*), of decalcified crab and crayfish cuticles, of decalcified cuttlefish and *Spirula* shell, and of the skeletal pen of the squid have been subjected to acidic (HCl) hydrolysis and the hydrolysates examined by ionophoresis and chromatography on paper. In every case very small amounts of aspartic acid and histidine were identified in addition to glucosamine and no other amino acids were present. Chitin samples, such as those mentioned, give a positive colour reaction with ninhydrin thus indicating the presence of a free amino group and possibly of a free amino group alpha to a carboxyl group.

Chitin has also been prepared from the larval cuticles of *A. spinicollis*, from the shell of the crab *S. serrata*, from the shell of cuttlefish, and from the skeletal pen of the squid (*Loligo* sp.) without resorting to digestion with hot aqueous alkali. Use was made of ethylenediaminetetra-acetic acid to decalcify the crab and cuttlefish materials and enzyme to remove protein from the *A. spinicollis* material. The skeletal pen of the squid was reprecipitated from aqueous lithium thiocyanate. The association of the chitin and protein in these four preparations is best considered when taken together with the results already published for the cuticle of the crab *Cancer pagurus* (Foster and Hackman 1957) and the larval cuticles of *A. spinicollis* (Hackman and Goldberg 1958). The protein contents of all these chitin preparations are given in Table 2.

The protein content of each of the chitin preparations given in Table 2 has been calculated from the nitrogen content of the chitin preparation. Chitin contains 6.9 per cent. nitrogen and the protein component has been considered to contain 16 per cent. nitrogen. Taking these calculated percentages of protein the results given in Table 1 have been expressed as mg amino acid obtained from 1 g protein. However, when these chitin preparations are hydrolysed an unknown amount of decomposition occurs because of the presence together of carbohydrate and amino acids. That this decomposition occurs is shown by the formation of a dark-coloured solution or sometimes of a black precipitate. Consequently the results given for the amino acid composition of each protein do not add up to 100 per cent. Moreover, since it is not known which amino acids have contributed to the formation of the products of decomposition it is not known which of the values given are low or by how much. Tryptophan, of course, has not been estimated. Nevertheless, certain conclusions can be drawn from the results and these are given below.

In all six chitin preparations it appears that chitin and protein are bound together by covalent bonds. The evidence for this conclusion is: (1) The methods of preparation exclude the presence of the more labile types of linkages such as would be broken by extraction with water, salt solution, aqueous urea, aqueous phenol, and other organic solvents. (2) The protein content of all the chitin preparations can be substantially reduced but the amino acid content has never been completely eliminated by repeated extraction with hot (100°C) aqueous alkali. (3) All the chitin preparations can be dispersed into aqueous lithium thiocyanate and the solutions on progressive dilution with acetone give a series of fractions

which contain both chitin and protein. In no case was a protein-free chitin fraction obtained. The presence of protein and chitin in the fractions was established by hydrolysing (HCl) each fraction and detecting, by ionophoresis and chromatography on paper, amino acids and glucosamine in the hydrolysates. Trim (1941) has reported that the puparia of the blowfly *Sarcophaga falculata* can be dispersed in aqueous lithium thiocyanate at 170°C and reprecipitated without effecting a separation of the chitin and protein components.

The observations made on the two types of chitin preparations (with or without the use of hot aqueous alkali) suggest that the protein of the cuticle is bound by covalent bonds to chitin so forming a glycoprotein or mucopolysaccharide. The difficulty experienced in removing the last traces of amino acids from the chitin by prolonged treatment with hot dilute alkali clearly shows how strongly the protein is bound to the chitin. The fractionations from lithium thiocyanate solutions indicate the polydisperse nature of the glycoproteins. The amount of protein covalently

TABLE 2  
PROTEIN CONTENT OF GLYCOPROTEINS CONTAINING CHITIN

Source of Glycoprotein	Percentage Protein (calc. from nitrogen content)
<i>Agrianome spinicollis</i> larval cuticle*	50
<i>Agrianome spinicollis</i> larval cuticle (papain)	5
<i>Scylla serrata</i> cuticle (EDTA)	15.6
<i>Cancer pagurus</i> cuticle (EDTA)†	7.5
Cuttlefish shell (EDTA)	51.2
<i>Loligo</i> sp. skeletal pen (lithium thiocyanate)	13.2

\* Hackman and Goldberg (1958).

† Foster and Hackman (1957).

bound to the chitin varies considerably. In the larval cuticles of *A. spinicollis* and in the cuttlefish shell about an equal weight of protein and chitin are bound together while in the cuticle of the crab *C. pagurus* the protein content of the glycoprotein is as low as 7.5 per cent. The protein content of the glycoproteins in the cuticle of *S. serrata* and the pen of *Loligo* are intermediate in value.

Nomenclature in the field of carbohydrate-protein complexes is in a state of flux and some recent classifications have been given by Kent and Whitehead (1955), Pigman (1957), and Bettelheim-Jevons (1958). According to the schemes outlined by Kent and Whitehead and by Bettelheim-Jevons the complexes formed from chitin and protein would be classified as either mucopolysaccharides or mucoproteins depending not only on the amounts of chitin and protein present but also on the "completeness of the protein structure". According to the scheme given by Pigman these covalently bound, stable combinations would be classified as glycoproteins. The results given in Table 2 show that the ratio of chitin to protein can vary greatly and so the less specific term, glycoprotein, as used by Pigman would appear to be preferable for these chitin-protein complexes. Moreover, the term glycoprotein has the added advantage of indicating a stable, covalently bound complex.

In this study chitins have been prepared from a number of different sources. The arthropods (insects and crustaceae) contained  $\alpha$ -type chitin and the squid skeletal pen contained  $\beta$ -type chitin. X-ray diffraction studies of the cuttlefish shell chitin showed that it was poorly orientated, that it differed in detail from  $\beta$ -chitin, and that it corresponded in general features to a third and as yet unnamed type of chitin (Rudall, personal communication). All chitins prepared by extraction of the cuticles (or decalcified cuticles) with hot aqueous alkali gave aspartic acid and histidine on acidic hydrolysis. However, differences do exist in the amino acid composition of the glycoproteins. The protein components of both the cuttlefish and squid glycoproteins have a higher glycine content than those of the crab or insect glycoproteins (Table 1). Of the amino acids estimated in the protein of the squid preparation glycine accounts for 13 per cent. by weight or 20 per cent. of the total number of amino acid residues present. For the cuttlefish protein component the figures are 8.6 per cent. and 14.1 per cent. respectively. Piez and Gross (1959) have shown that in invertebrate and vertebrate collagens one-third of the total number of amino acid residues are glycine. The squid and cuttlefish protein components do not contain this amount of glycine and in view of the absence of hydroxyproline these proteins cannot be described as being of the collagen type. Cysteine or cystine or both are present in the protein components of the cuttlefish and squid glycoproteins but are absent from the chitin-containing glycoproteins of arthropods. Although there are similarities in the amino acid composition of the glycoproteins from cuttlefish shell and squid skeletal pen there are two marked differences, namely the higher aspartic acid and tyrosine content of the former. The protein components of both arthropod glycoproteins have a high aspartic acid and tyrosine content.

Although glycoproteins, in which the carbohydrate fraction is non-acidic, have been described from many sources very little is known of the nature of the bond or bonds linking the protein to the carbohydrate. Johansen, Marshall, and Neuberger (1958) obtained a peptide-carbohydrate complex from egg albumin which contained mannose, glucosamine, leucine, and aspartic acid. As a result of degradative experiments they concluded that an aspartic acid residue is linked directly to the carbohydrate through one of its carboxyl groups. Glycoproteins of similar composition have been obtained from the same source by Cunningham, Nuenke, and Nuenke (1957) and Jevons (1958). Rosevear and Smith (1958) isolated a glycoprotein from human  $\gamma$ -globulin in which they concluded that the peptide was linked with the carbohydrate residue through the  $\beta$ -carboxyl group of aspartic acid. It is probable that these links are of the amide or ester type.

The results described above for the chitin-containing glycoproteins indicate that the protein is linked to chitin through aspartyl or histidyl residues or both. In view of the stability of the link to hot alkali and its instability to hot acid this link could be in the form of an *N*-acylglucosamine. The *N*-acetyl link in chitin shows this type of behaviour although the acetyl group of *N*-acetyl-D-glucosamine is lost when heated with acid or alkali. Attempts to identify free amino groups, by the dinitrofluorobenzene method, in the chitins prepared by extraction of the cuticles with hot alkali were not successful. Because an unknown amount of decomposition occurs during the acid hydrolysis of these glycoproteins it is not possible to determine accurately the ratio of amino acids to glucosamine. However, an estimate was



obtained of the amino acids in the chitin prepared by alkaline extraction of the larval cuticles of *A. spinicollis*, it being one amino acid residue per 200–300 glucosamine residues. Although the length of the chitin chain is not known it is generally considered to consist of several hundred *N*-acetyl-D-glucosamine residues and so it is possible that only one or two protein chains are linked to each chitin chain.

The work described above makes it probable that chitin does not occur uncombined with protein. This raises the question of terminology. Chitin, by common usage, refers to a polymer composed only of *N*-acetyl-D-glucosamine residues. It is suggested that for convenience the glycoproteins, the chitin-protein complexes, be referred to as "native chitin" and a distinction is therefore made between "native chitin" and "chitin". Since the chitin-containing glycoprotein is polydisperse one or more of the glycoproteins may be more readily attacked by the enzyme chitinase. Jeuniaux (1959) has described a membrane in the cuticle of crustacea which is resistant to the action of chitinase and it contains 73 per cent. chitin and 12–15 per cent. protein. Removal of the protein by alkaline digestion permits the chitin to be degraded by the enzyme. The peritrophic membrane of insects may represent another chitin-containing glycoprotein which is not attacked by the enzyme chitinase.

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# PATHOLOGY OF INFESTATION OF THE RAT WITH *NIPPOSTRONGYLUS MURIS* (YOKOGAWA)

## V. PROTEIN DIGESTION

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### Summary

The digestion of protein by rats infested with the intestinal nematode *Nippostrongylus muris* (Yokogawa, 1920) was measured directly by feeding by stomach tube egg albumin labelled with radioiodine. One hour later nearly 70 per cent. was recovered from the gastro-intestinal tract of infested rats, but only 50 per cent. from that of normal rats. This difference was due largely to a depression of digestion in the small intestine, although absorption was also slightly reduced. Gastric digestion was not affected, nor was there any difference between the rates of gastric emptying by normal and infested rats.

## I. INTRODUCTION

General texts on parasitology commonly refer to the possible effects of intestinal nematode infestation upon digestion by the host. Lapage (1956), for instance mentions the possible effect of *Trichostrongylus* spp. upon digestion and absorption but does not refer to observations or experiments. Several investigators have made indirect assessments by balance studies. Stewart (1933), Shearer and Stewart (1933), and Franklin, Gordon, and Macgregor (1946) have shown by this method that there was decreased digestion of crude protein and decreased net mineral absorption by sheep infested with various species of nematodes. Spedding (1954) and Shumard, Bolin, and Eveleth (1957) confirmed that the digestibility of crude protein was decreased by infestation, but Andrews, Kauffman, and Davis (1944) could not confirm that either digestion or absorption was affected in sheep infested by *T. colubriformis*. Rogers (1941, 1942) has shown that rats infested with *Trichinella spiralis* experienced an impaired protein digestion during the intestinal phase.

It is not possible to separate digestion from absorption, nor to allow for secretion into the lumen of the gut when digestion is measured by means of balance studies. It is conceivable that only one of these may be deranged. For these reasons a direct measurement of protein digestion was of considerable interest in nippostrongylosis of rats and in intestinal infestations generally.

Egg albumin labelled with radioiodine was used to measure protein digestion. The efficacy of this method has been discussed by Borgstrom *et al.* (1957). The rate of gastric emptying and absorption from the small intestine was also estimated. An approximate comparison between normal and infested rats was also made of gastric digestion as distinct from digestion in the stomach and small intestine together.

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## II. METHODS

The type of rats used, the method of infesting them, and the reasons for carrying out the experiments on the tenth day of the infestation have been stated earlier by Symons (1957). A 1 per cent. (w/v) solution of a technical grade of egg albumin (E.A.) in 0.9 per cent. NaCl was labelled with  $^{131}\text{I}$  by the ammoniacal method of Francis, Mulligan, and Wormall (1959). Before being fed to the rats, sufficient carrier E.A. was added to this solution to increase the protein concentration to about 5 per cent. The rats which were fasted overnight were fed 2 ml of this solution by stomach tube, without anaesthesia, using the gagging device of Gillespie and Lucas (1957). They were then returned to their cages for 1 hr, after which they were killed by a blow on the head. Infested rats were fed and killed alternately with normal controls.

The stomach and small intestine were isolated by ligatures. The stomach was opened and washed directly into a 25-ml volumetric flask with cold 0.25 per cent. E.A. The small intestine was opened longitudinally into a 100-ml conical flask and washed six times. The washings were transferred successively to a 100-ml volumetric flask. The caecum and large bowel were discarded, as it was determined previously that less than 1 per cent. of the radioactivity of the preparation administered reached these organs in either infested or normal rats 1 hr after feeding. The flasks were stored at about 4°C for 1–2 hr, after which 5-ml samples were transferred to graduated centrifuge tubes and the protein precipitated by the addition of 5 per cent. phosphotungstic acid to a final concentration of 2.5 per cent. (Borgstrom *et al.* 1957). After a further 1–2 hr at about 4°C the precipitates were spun down. When washings from the stomach or small intestine were added to the labelled E.A. solution and the protein precipitated, less than 1 per cent. of the radioactivity remained in the supernatant.

After the volume of the supernatant was recorded, it was separated from the precipitate, which was drained. Triplicate 2-ml aliquots of the supernatant were placed in suitable test tubes for radio-assay. The precipitates were dissolved in about 5 ml of 0.4N NaOH, then diluted with water to 10 ml and 2-ml aliquots taken for counting. The radioactivity was expressed as counts/sec in all instances.

The radioactivity in the 2 ml of E.A. solution fed to the rats was estimated by diluting samples with 0.25 per cent. E.A. and taking 2-ml aliquots for counting. In addition, 5-ml aliquots of these diluted solutions were precipitated with phosphotungstic acid to estimate any free  $^{131}\text{I}$  which was present in the original solution and allowance was made for this in the final calculations.

All count rates were determined with a well-type EKCO N550A scintillation counter, and an EKCO N530 automatic scaler, and were corrected for background and decay.

Calculations were made assuming that labelled and carrier protein were not digested or absorbed differently, and that all the radioactivity in the supernatant represented completely or partially hydrolysed protein.

The radioactivity in the worms was estimated to ensure that the amount of protein or the products of hydrolysis, which was taken up by the parasites themselves, was not of significance to the experiment. The parasites were recovered

from three rats, 1 hr after feeding 2 ml of labelled E.A., by opening the intestines longitudinally into warm physiological saline in conical flasks. The intestines were allowed to remain in the flasks for a few minutes before removal. The parasites were then allowed to settle. They were washed at least seven times with saline, the excess water was removed on filter paper, and the worms spread thinly on planchettes. In this instance the radioactivity present in the parasites per mg of wet weight, and in an appropriate dilution of the E.A. solution fed to the rats, was then determined using a Geiger-Müller tube with a thin end-window.

### III. RESULTS

The activity taken up by the parasites was found to be less than 0.01 per cent. of the total activity fed to the rats and was therefore ignored in all calculations.

There were nine rats in both the normal and in the infested groups, but one of the latter had to be discarded as a tear was found in the small intestine. The severity of the infestations, judged subjectively, was found to range from light to heavy; four infestations were heavy.

The sum of digested and undigested protein recovered from the gastro-intestinal tract, expressed as a percentage of the total radioactivity fed to the rats, was calculated from the equation

$$R_a = [(S_a + I_a)/T_a] \times 100,$$

where

$R_a$  = percentage radioactivity recovered,

$S_a + I_a$  = sum of the activities of the supernatant and precipitated protein from the stomach and small intestine respectively, and

$T_a$  = total radioactivity fed to each rat.

The proportion digested in the stomach and small intestine together was expressed as a percentage of the total radioactivity fed to an animal and was calculated from the equation

$$\text{Percentage digestion} = \{[T_p - (S_p + I_p)]/T_p\} \times 100,$$

where  $S_p$ ,  $I_p$ , and  $T_p$  are the activities in the precipitated protein from the stomach, small intestine, and meal fed to the rats respectively. The last of these three quantities was smaller than the total activity fed to the rats ( $T_a$ ) because allowance was made for free  $^{131}\text{I}$  in the solution.

The radioactivity available for absorption from the small intestine (expressed in terms of the symbols used above) was  $T_a - (S_a + I_p)$  and the radioactivity actually absorbed was  $T_a - (S_a + I_a)$ . The percentage absorbed from the small intestine was then calculated from the equation

$$\text{Percentage absorbed} = \frac{(T_a - S_a) - I_a}{(T_a - S_a) - I_p} \times 100.$$

The results of the experiment are shown in Table 1. Fifty per cent. of the meal was recovered from the normal and 68.9 per cent. from the infested rats. The difference between these two, the 95 per cent. confidence limits of which are also



shown in the table, is due mainly to a depression of digestion, although absorption from the small intestine was also reduced. It is emphasized that digestion here refers to the sum of digestion in the stomach and small intestine. In normal rats 58.6 per cent. of the meal was digested while only 35.8 per cent. was digested by the infested animals. The difference within the 95 per cent. limits of confidence indicates that this is a well-marked depression. The lower percentage of absorption by the small intestine, as can be seen from the table, is not so clearly significant.

Gastric emptying was not affected by the infestation as about 90 and 91 per cent. of the meal left the stomach in 1 hr in normal and infested rats respectively. An approximate comparison of gastric digestion as distinct from digestion in the stomach and small intestine together could be made by calculating the ratio of hydrolysed protein to undigested protein that remained in the stomach at the time of death. There was no statistical difference between gastric digestion by normal and infested rats.

TABLE 1

RECOVERY, DIGESTION, AND ABSORPTION OF EGG ALBUMIN IN RATS INFESTED WITH *N. MURIS*

	Normal	Infested	Difference*
Total recovery from gastro-intestinal tract (as % of total radioactivity fed)	50.0	68.9	$-18.9 \pm 10.6$
Digestion in stomach and small intestine (as % of radioactivity fed as labelled protein)	58.6	35.8	$22.8 \pm 13.9$
Absorption from small intestine (as % of radioactivity available for absorption)	89.6	80.3	$9.3 \pm 8.7$
Gastric digestion (ratio: $\frac{\text{hydrolysed protein}}{\text{undigested protein}}$ )	0.64	1.21	$-0.57 \pm 1.05$
Gastric emptying (as % of radioactivity fed)	89.5	90.7	$-1.2 \pm 11.1$

\* Differences are given with their 95 per cent. confidence limits.

#### IV. DISCUSSION

Advantages of this method are that the labelled protein can be distinguished from the endogenous protein secreted into the gut during digestion, and absorption and digestion can be separated. Dreisbach and Nasset (1954) have demonstrated that the amount of nitrogenous material recovered from the gastro-intestinal tract of rats was equal to, or even greater than, the amount of protein ingested. Pisano, Paine, and Taylor (1959) found that the nitrogen secreted into the duodenal loop of chickens obscured the measurement of absorption of protein from that region.

In the experiment reported here, about 70 per cent. of the radioactivity was recovered from the gastro-intestinal tract of the infested rats, while only 50 per cent. was recovered from controls. A reduced ability to digest the egg albumin was largely responsible for this difference, but there also appeared to be a derangement of absorption. The impairment of egg albumin digestion supports the findings of the earlier workers mentioned, who by balance studies concluded that nematode infestations in sheep or rats could depress protein digestion. Because there was no impairment of gastric digestion it appears that this depression of digestion associated



with nipprostrongylosis occurs entirely in the small intestine, which is the site of infestation. The secretion of anti-enzymes by the parasite was postulated by Shearer and Stewart (1933) to inhibit the action of pepsin, but the present experiment offers no evidence as to the cause of the depression.

The possible reduction of absorption of nitrogen from the small intestine as a whole is interesting. The values reported here have not strong statistical support, but they tend to contradict the experiments reported in Part IV of this series (Symons 1960). It was then found that neither glucose nor histidine absorption was retarded in the complete small intestine of the infested rats; on the other hand, perfusion of the infested jejunum alone did show that glucose absorption from that section was reduced to one-third or less of the normal rate, but histidine absorption was not tested in the jejunum alone. Borgstrom *et al.* (1957) found that, in man, although absorption of fat, carbohydrate, and protein began in the duodenum, protein absorption was not complete even at the distal end of the small intestine; furthermore, the absorption of glucose was completed in the proximal half of that organ. These findings support those of Schlüssel and Sunder-Plassmann (1953) who reported that some of the protein labelled with radiosulphur and fed to rats was recoverable from the rectum, although the greatest degree of absorption occurred in the duodenum and upper jejunum. This slower rate of protein than glucose absorption may mean that the jejunum, which is the site of the infestation, is relatively more important to the former and could account for the discrepancy between this and the earlier experiment. In addition, there may be a complex interrelationship between the rate of digestion, the concentration of the products of hydrolysis, and absorption, which was not present in the earlier experiments.

Neither the depression of digestion nor of absorption was likely to be due to increased motility of the upper part of the small intestine as Symons (1959) has shown that there is, in fact, a significant decrease in the rate of passage through this part of the gut of infested rats.

Both groups of animals passed about 90 per cent. of the meal from their stomachs in 1 hr. This confirms earlier experiments (Symons 1959, 1960) when it was found that infested rats emptied a solid meal or solution of glucose or histidine from the stomach at the same rate as the controls. The estimate of the rate of gastric emptying makes no allowance for the fact that some of the meal fed by stomach tube is forced past the pylorus nor for any regurgitation into the stomach that may occur. This regurgitation which, because of the greater volume of ingesta in the upper small intestine (Symons 1957), could conceivably be more pronounced in the infested rats, would also interfere with the measurement of protein digestion in the stomach. For this reason, the estimates of gastric emptying and digestion must be accepted with caution.

#### V. ACKNOWLEDGMENTS

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# THE PRE-NATAL DEVELOPMENT OF SKIN AND HAIR IN CATTLE\*

II. *BOS INDICUS* L.  $\times$  *B. TAURUS* L.

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## Summary

A histological study has been made of the development of the skin and hair follicles in 22 crossbred (Brahman  $\times$  Hereford or Brahman  $\times$  Shorthorn) cattle fetuses, ranging in age from 77 to 263 days, and in three new-born calves.

Skin samples were examined from the head, midside, and back regions of the fetuses, but from the midside region only of the new-born calves.

The initiation of hair follicles begins at about the 78th day of gestation in all regions. It continues to about the 140th day on the head, about the 160th day on the back, and about the 180th day on the midside.

Three follicle types (first-, later-, and last-formed) are recognized. The first-formed follicles develop at about the same rate in the three regions studied, but they are slower than all the later- and last-formed follicles. All follicle types on the head are slightly more advanced than those on the trunk positions.

Up to about the 140th day of gestation the follicle density is greatest on the head. The density of the follicles on the midside resembles that found in *B. taurus*.

No difference in skin thickness between *B. indicus*  $\times$  *B. taurus* and *B. taurus* can be detected.

## I. INTRODUCTION

In a previous paper (Lyne and Heideman 1959) on the pre-natal development of skin and hair in *Bos taurus* attention was drawn to the need for further pre- and post-natal histological studies of the integument of different breeds of cattle.

It is the aim of this paper to give such an account of the pre-natal development in *B. indicus*  $\times$  *B. taurus*, and to make a comparison of some of its features with those of *B. taurus*.

## II. MATERIAL AND METHODS

### (a) Animals

Details of the animals sampled are shown in Table 1. The dams (Hereford and Shorthorn) were mated with one Brahman (Zebu) bull and were maintained at the National Cattle Breeding Station, Rockhampton, Qld. Although the Brahman breed is derived from Indian breeds with probably some European blood (Mason 1951), the crossbreds examined in the present study are referred to as *B. indicus*  $\times$  *B. taurus*.

The body weights of all specimens sampled were obtained before fixation in formalin.

\* For Part I in this series see *Aust. J. Biol. Sci.* **12**: 72.

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(b) *Skin Sampling and Histological Methods*

Initially, two skin samples were taken from the midside (mid-lateral region of the trunk) of the animals and were fixed in 5 per cent. formol saline. The fetuses were then preserved in 10 per cent. formalin and later some additional samples were taken from the head (midline between the eyes) and the back (posterior mid-dorsal region immediately in front of the tail). Additional samples (referred to as later samples) were also taken from the midside in order to make a comparison of the follicle and hair populations in samples taken at the same time from the different body regions. The samples were taken with 1-cm or, where this was not possible, with 0.5-cm dia. trephines. After paraffin embedding, serial sections were cut at  $8\ \mu$  parallel to the skin surface. Some additional rectangular skin samples were cut

TABLE 1  
DETAILS OF *B. INDICUS* × *B. TAURUS* SPECIMENS SAMPLED

Specimen No.	Foetal Age (days)	Sex	Breed	Specimen No.	Foetal Age (days)	Sex	Breed
B50	77	♀	H*	B63	143	♀	S
B48	78	♀	S†	B65	160	♀	S
B49	78	♀	S	B66	160	♀	H
B46	79	♀	H	B67	178	♂	H
B47	84	♂	H	B68	180	♀	S
B52	100	♀	H	B69	198	♂	H
B51	102	♂	H	B70	209	♂	S
B53	102	♂	S	B71	238	♀	H
B58	123	♂	H	B72	263	♀	H
B60	125	♂	S	B74	281 + 4‡	♀	S
B59	126	♂	S	B73	288 (birth)	♂	S
B61	141	♀	H	B75	289 (birth)	?	H
B62	141	♂	H				

\* Brahman × Hereford.

† Brahman × Shorthorn.

‡ Four days old when sampled.

at 8, 15 or 20, and  $40\ \mu$  parallel to the long axes of the follicles. Staining was with haemalum, eosin, and picric acid. Haemalum-stained whole mounts of skin from fetuses up to 102 days of age were also examined.

(c) *Skin and Follicle Measurements*

(i) *Macroscopic Measurements.*—After fixation the mean skin thickness (excluding the panniculus carnosus) of four samples at each position was estimated from measurements made with an instrument (Wodzicka 1958) which exerted a constant pressure of 50 g/cm<sup>2</sup>. Where necessary hair was closely clipped.

To allow comparison with the present material, most of the *B. taurus* skin samples measured by Lyne and Heideman (1959) were remeasured with this instrument. Although all samples had been fixed in 10 per cent. formalin they had been stored in the fixation fluid for different periods (1–2½ yr) when the skin thickness measurements were carried out.



TABLE 2  
SUMMARY OF STAGES IN THE DEVELOPMENT OF THE DIFFERENT TYPES OF HAIR FOLLICLES IN B. INDICUS × B. TAURUS

Specimen No.	Foetal Age (days)	Stage of Most Advanced First-formed Follicles			Stage of Most Advanced Later-formed Follicles			Stage of Least Advanced Last-formed Follicles			Stage of Least Advanced Follicles		
		Head	Midside	Back	Head	Midside	Back	Head	Midside	Back	Head	Midside	Back
B50	77	?	1a	—	—	—	—	—	—	—	?	1a	—
B48	78	1a	1a	1a	—	—	—	—	—	—	1a	1a	1a
B49	78	1a	1a	—	—	—	—	—	—	—	1a	1a	—
B46	79	1a	1a	1a	—	—	—	—	—	—	1a	1a	1a
B47	84	1a	1a	?	—	—	—	—	—	—	1a	1a	?
B52	100	2a	1b	1b	—	—	—	—	—	—	1a	1a	?
B51	102	2a	1b	1b	—	—	—	—	—	—	1a	1a	1a
B53	102	2a	1b	1b	—	—	—	—	—	—	1a	1a	1a
B58	123	2a	2a	2a	2a	—	—	—	—	—	1a	1a	1a
B60	125	2a	2a	2a	2a	1b	—	1a	—	—	1a	1a	1a
B59	126	2b	2a	2a	2a	—	—	1a	—	—	1a	1a	1a
B61	141	2b	2a	2b	2a?	1b	2a	1b	—	1a	1b	1a	1a
B62	141	2b	2a	2a	2a	1b	2a	1a	—	1a	1a	1a	1a
B63	143	3a	2b	3a	3a	1b-2a?	2a	2a	—	1a	1a	1a	1a
B65	160	3a	2b	3a	3a	2a	2a	2a	1a	1a	2a	1a	1a
B66	160	3a	2b	3a	3a	2a	3a	2a	1a	1b	2a	1a	1b
B67	178	3a	3a	3a	3b	2a	3a	3a	1a	1b	3a	1a	1b
B68	180	4	3b	3b	3b	2b	3b	3a	1a	2a	2b*	1a	2a
B69	198	6	4	3b	3a-3b	3a	3b	3a	1a	2a	3a*,†	1a	2a
B70	209	6	6	6	8-9	3b	6	6	3a	5-6	3b*	3a,	3b*
B71	238	9	6	9	8-9	4	8	7	3a	7	4*	3a	4*
B72	263	10c	10c	10a	10c	10c	10c	10b	5	10c	8-9*	5†	9*,†,†
B74	281+4§	?	10c	?	?	10c	10c	10c	10c	10c	9*	9*	9*
B73	288	?	10c	?	?	10c	?	?	?	?	?	?	?
B75	289	?	10c	?	?	5¶	?	?	10c	?	?	10a*	?

\* First-formed follicles. † Later-formed follicles. ‡ Last-formed follicles. § Four days old when sampled. || Birth samples. ¶ Second cycle.

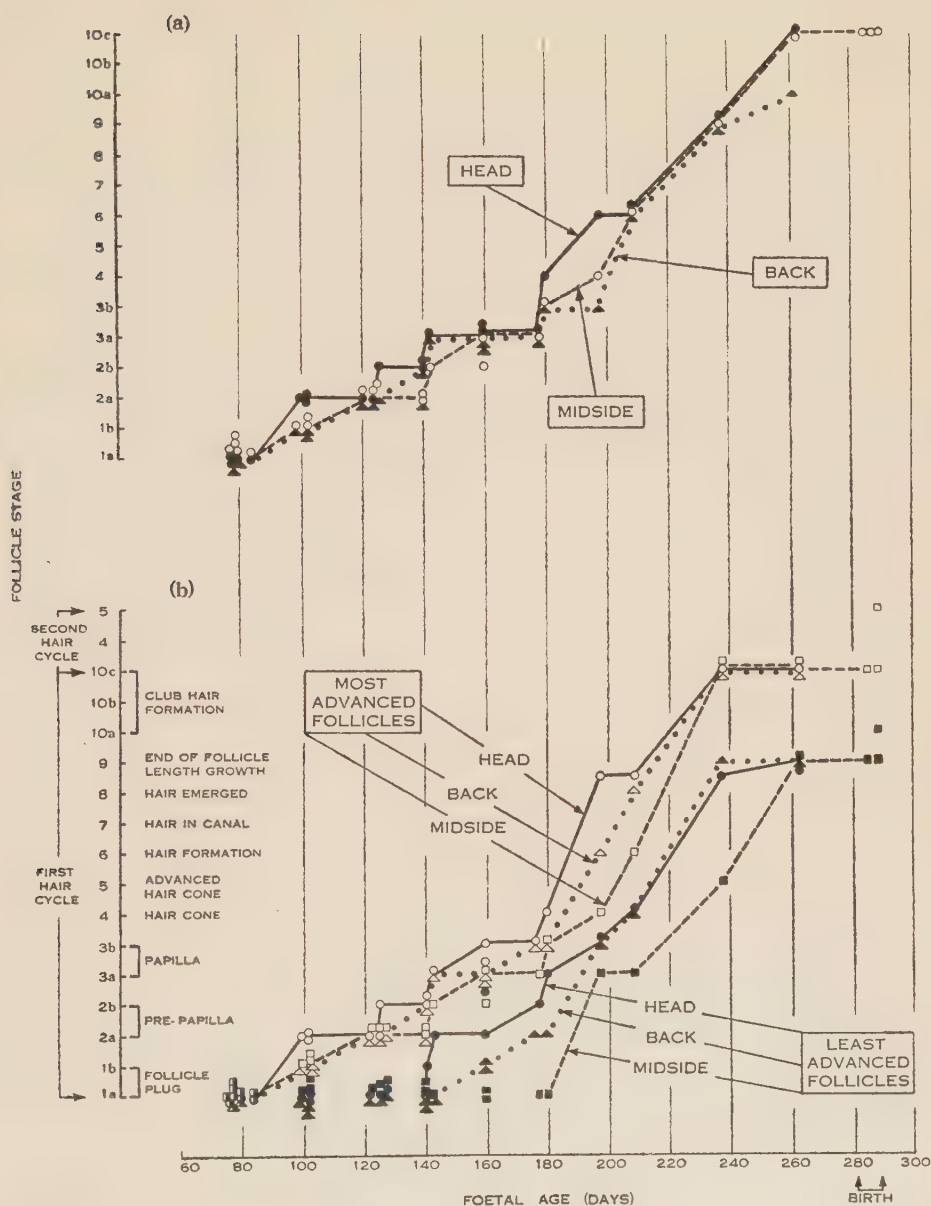


Fig. 1.—(a) Relation between foetal age and stage of development of the first-formed hair follicles on the head, midside, and back in *B. indicus* x *B. taurus*. (b) Relation between foetal age and stage of development of the most advanced and least advanced follicles (irrespective of type) on the head, midside, and back in *B. indicus* x *B. taurus*.

(ii) *Microscopic Measurements*.—Microscopic measurements of the dimensions of the hair follicles and associated structures and counts of the follicle and hair populations were made at a magnification of  $\times 215$ , as described by Lyne and Heideman (1959).

### III. RESULTS

#### (a) *Development of Individual Follicles*

(i) *Stages of Development*.—The stages of hair follicle development, described for *B. taurus* by Lyne and Heideman (1959), are easily identified in the present material and all follicle types (first-, later-, and last-formed) follow the same developmental pattern. The first-formed follicles are associated with a pair of later-formed follicles situated on either side and at right angles to the ectal-ental axis. The last-formed follicles form groups associated with those formed earlier.

(ii) *Rate of Development*.—The relation between foetal age and stage of development of the different types of follicles is shown in Table 2 and Figures 1 and 2. Figure 1(a) shows the rate of development of the most advanced of the first-formed follicles in the three positions examined. These follicles begin their development at about the same foetal age (the 78th day of gestation) and progress at the same rate throughout the entire first hair cycle, which has been completed by birth. The first-formed follicles on the head are slightly more advanced than those on the two trunk positions, where the rates of development are very similar.

Figure 1(b) illustrates the rate of development of the most advanced and least advanced follicles (irrespective of follicle type) in the three positions. It is evident that the most advanced follicles are usually found on the head. In several of the older fetuses the follicles on the back are slightly ahead of those on the midside.

Considering the least advanced follicles, it is seen that on the head follicle initiation ceases at about the 140th day, on the back at about the 160th day, while on the midside new follicles are being formed until about the 180th day. Up to about the 200th day the rate of development of the least advanced follicles on the back is intermediate between that on the head and midside.

Figure 2(a) shows the relation between foetal age and stage of development of the three types of follicles on the head. The first-formed follicles develop at a slower rate than all the later- and last-formed follicles. The approximate range of developmental stages reached by the first-formed follicles is shown. The last of the last-formed follicles to be initiated develop at the fastest rate but at no stage do they quite overtake the most advanced of the later-formed type. At about the 200th day all the first-formed follicles have been overtaken by both later- and last-formed follicles and these therefore reach the end of the first hair cycle (stage 10c) before the first-formed follicles.

Figure 2(b) shows the similarity in rate of development of the first-formed follicles in *B. taurus* (Lyne and Heideman 1959) and in *B. indicus*  $\times$  *B. taurus* as seen on the midside region. The stage-age curves coincide up to about the 180th day. Thereafter, the rate of development up to stages 8–9 is slightly faster in *B. taurus* but there is not sufficient material to establish breed differences. The later- and last-formed follicles develop at closely similar rates in the two breed types.

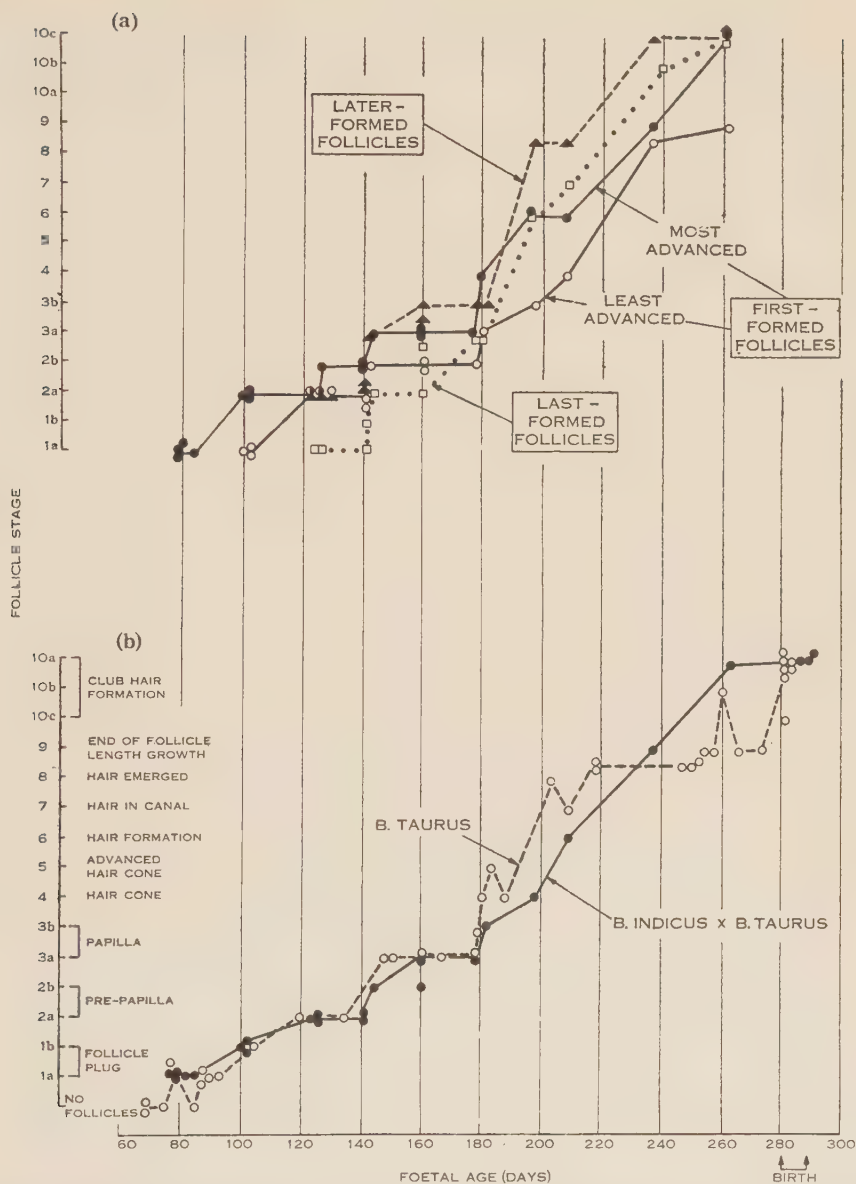


Fig. 2.—(a) Relation between foetal age and stage of development of different types of hair follicles (most advanced of the first- and later-formed follicles; least advanced of the last-formed) on the head in *B. indicus* × *B. taurus*. The approximate stage of development of the least advanced of the first-formed follicles is also shown. (b) Relation between foetal age and stage of development of the most advanced of the first-formed follicles on the midside in *B. indicus* × *B. taurus* and *B. taurus* (Lyne and Heideman 1959).



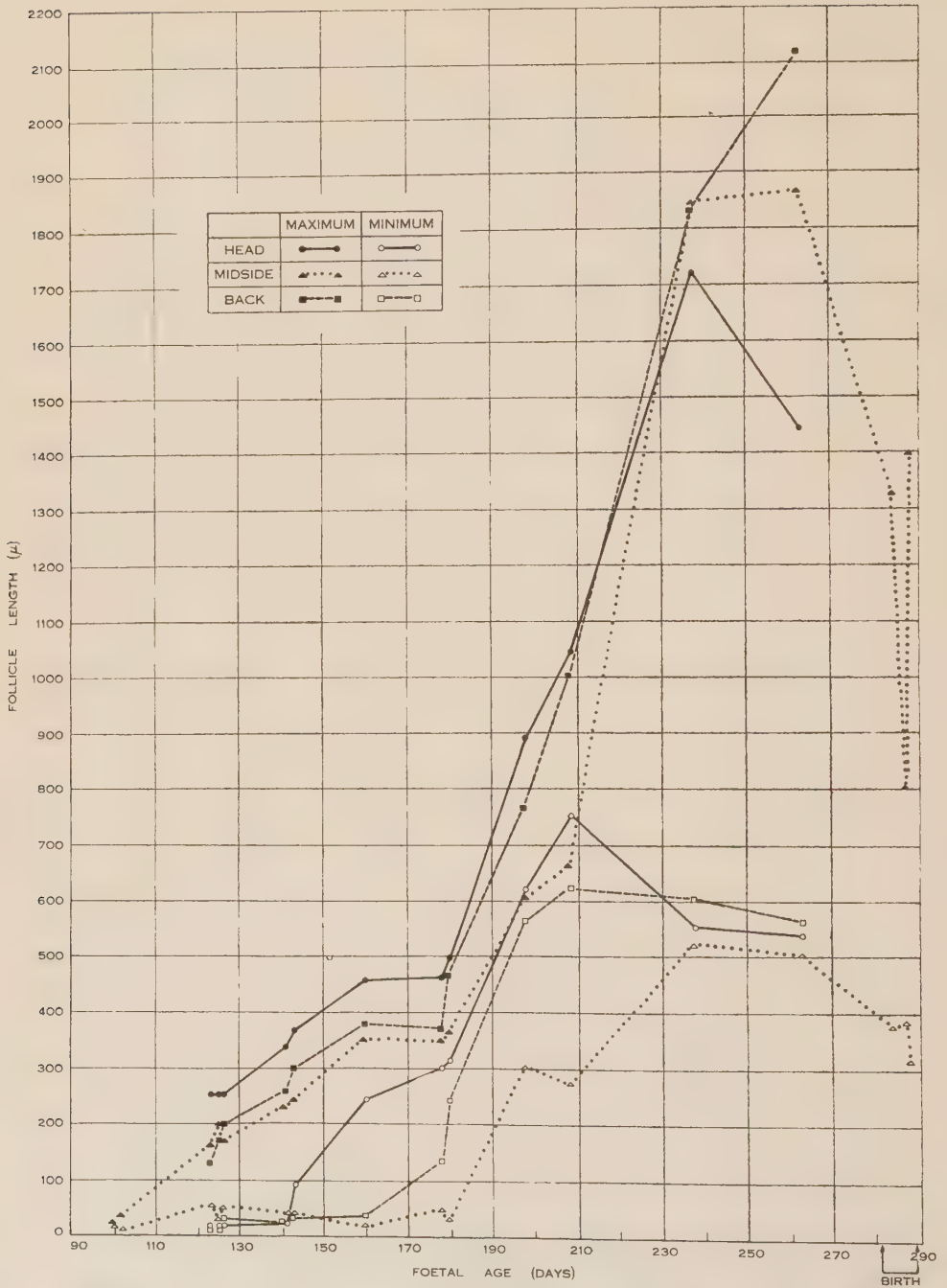


Fig. 3.—Relation between foetal age and maximum and minimum lengths of all follicles measured in *B. indicus* × *B. taurus*. The number of follicles measured is given in Table 3.

Follicle initiation in *B. taurus* ceases at about the 166th day (Lyne and Heideman 1959) whereas in *B. indicus* × *B. taurus* early follicle plugs (stage 1a) are still apparent at the 180th day (Table 2).

(iii) *Relation between Foetal Age and Follicle and Sweat Gland Length.*—The relation between foetal age and the maximum and minimum lengths of all the hair

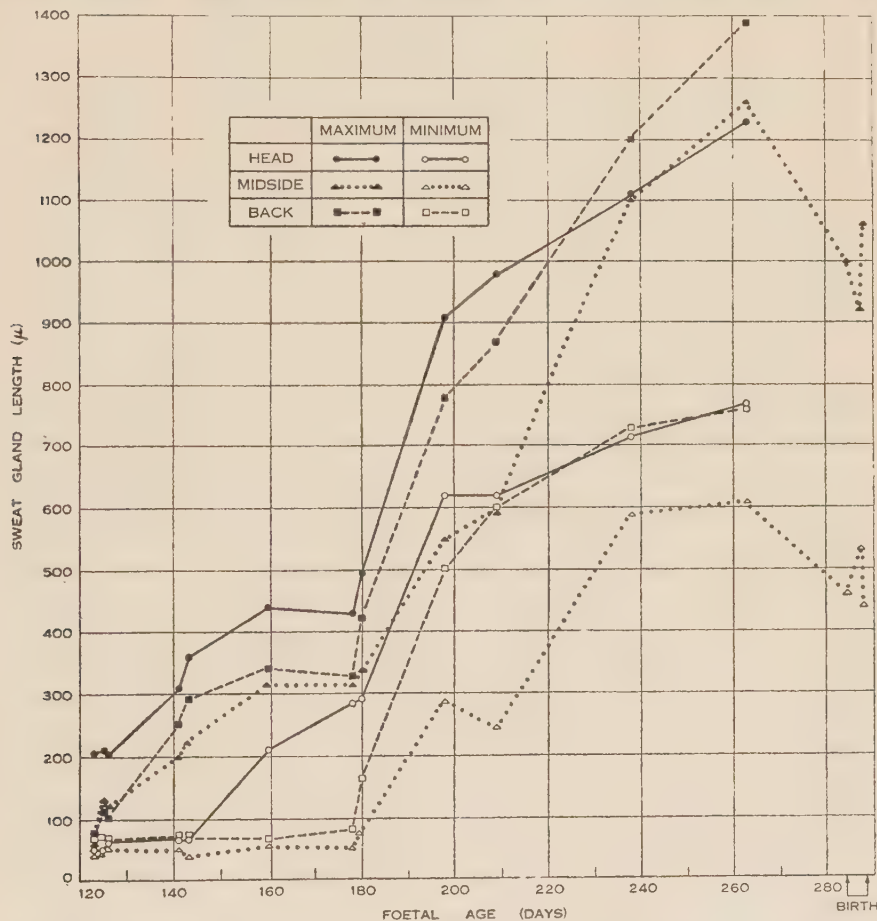


Fig. 4.—Relation between foetal age and maximum and minimum lengths of all sweat glands measured in *B. indicus* × *B. taurus*. The number of sweat glands measured is given in Table 3.

follicles and sweat glands measured is shown in Figures 3 and 4. Mean follicle and sweat gland lengths and the number measured are shown in Table 3.

In general, the follicles are longest on the head and shortest on the midside. The longest (first-formed) follicles reach their maximum length of 1700–2100  $\mu$  between the 240th and 260th day. At birth, the maximum length on the midside, the only position examined, is about 1400  $\mu$ . This is because many of the longest follicles have reached the end of the first hair cycle, and this is accompanied by a decrease in follicle length.

The maximum sweat gland length (1200–1400  $\mu$ ) is reached at the 263rd day. At birth, on the midside, the maximum length is about 1000  $\mu$ ; sweat glands were not measured on the other regions at birth.

(iv) *Relation between Follicle Stage and Follicle and Sweat Gland Length.*—It is clear (Fig. 5) that on the head and back regions, follicles at stages 3, 4–5, and 6–7 are very similar, while at the same stages, follicles on the midside are much shorter.

TABLE 3  
MEAN FOLLICLE AND SWEAT GLAND LENGTHS IN *B. INDICUS*  $\times$  *B. TAURUS*  
Number measured shown in parenthesis

Specimen No.	Foetal Age (days)	Mean Follicle Lengths ( $\mu$ )			Mean Sweat Gland Lengths ( $\mu$ )		
		Head	Midside	Back	Head	Midside	Back
B52	100	— —	20 ( 5)	— —	— —	— —	— —
B51	102	— —	17 ( 8)	— —	— —	— —	— —
B53	102	— —	27 ( 6)	— —	— —	— —	— —
B58	123	93 (48)	109 (29)	59 (48)	101 (14)	56 (13)	— —
B60	125	98 (36)	125 (48)	76 (48)	114 (13)	80 (27)	83 ( 8)
B59	126	112 (48)	121 (17)	107 (44)	114 (19)	67 (13)	77 (10)
B61	141	136 (57)	126 (48)	142 (50)	126 (21)	106 (27)	145 (28)
B62	141	141 (63)	138 (46)	126 (48)	148 (29)	116 (30)	129 (25)
B63	143	212 (47)	134 (50)	149 (51)	193 (36)	143 (26)	154 (32)
B65	160	326 (49)	118 (64)	131 (49)	318 (47)	138 (32)	133 (19)
B66	160	321 (58)	167 (48)	222 (58)	323 (40)	146 (42)	235 (20)
B67	178	354 (50)	144 (48)	234 (54)	352 (34)	135 (33)	180 (46)
B68	180	385 (50)	191 (79)	317 (50)	411 (30)	216 (25)	285 (38)
B69	198	782 (48)	401 (88)	653 (49)	731 (26)	381 (56)	643 (24)
B70	209	888 (79)	382 (94)	865 (57)	782 (60)	353 (61)	716 (38)
B71	238	963 (61)	879 (79)	889 (61)	880 (41)	725 (31)	859 (35)
B72	263	695 (25)	820 (79)	873 (63)	918 (14)	810 (61)	958 (29)
B74	281 + 4*	— —	594 (105)	— —	— —	677 (58)	— —
B73	288	— —	541 (92)	— —	— —	718 (56)	— —
	(birth)						
B75	289	— —	559 (88)	— —	— —	737 (53)	— —
	(birth)						

\* Four days old when sampled.

As in *B. taurus* (Lyne and Heideman 1959) the end of the active growth of the hair (stage 10a) and the formation of a keratinized club hair (stage 10c) is marked by a decrease in follicle length. Up to stages 6–7 sweat glands (Fig. 5) are longest on the head and shortest on the midside.

(v) *Relation between Sweat Gland Length and Follicle Length.*—Figure 6 shows the relation between sweat gland length and follicle length (up to stage 9) in the three regions examined. The sweat glands are appendages of the follicles and start growing a little later. In their early stages of development the sweat glands grow faster than their follicles, especially on the head, so that sweat gland length soon equals follicle

length. Later the growth of the sweat glands lags behind the growth of the follicles so that almost all sweat glands end up being shorter than their associated follicles. This does not entirely hold for the head where some of the sweat glands keep growing at a faster rate for a longer period than those of the other follicles. Most of these sweat glands belong to follicles of the first-formed type.

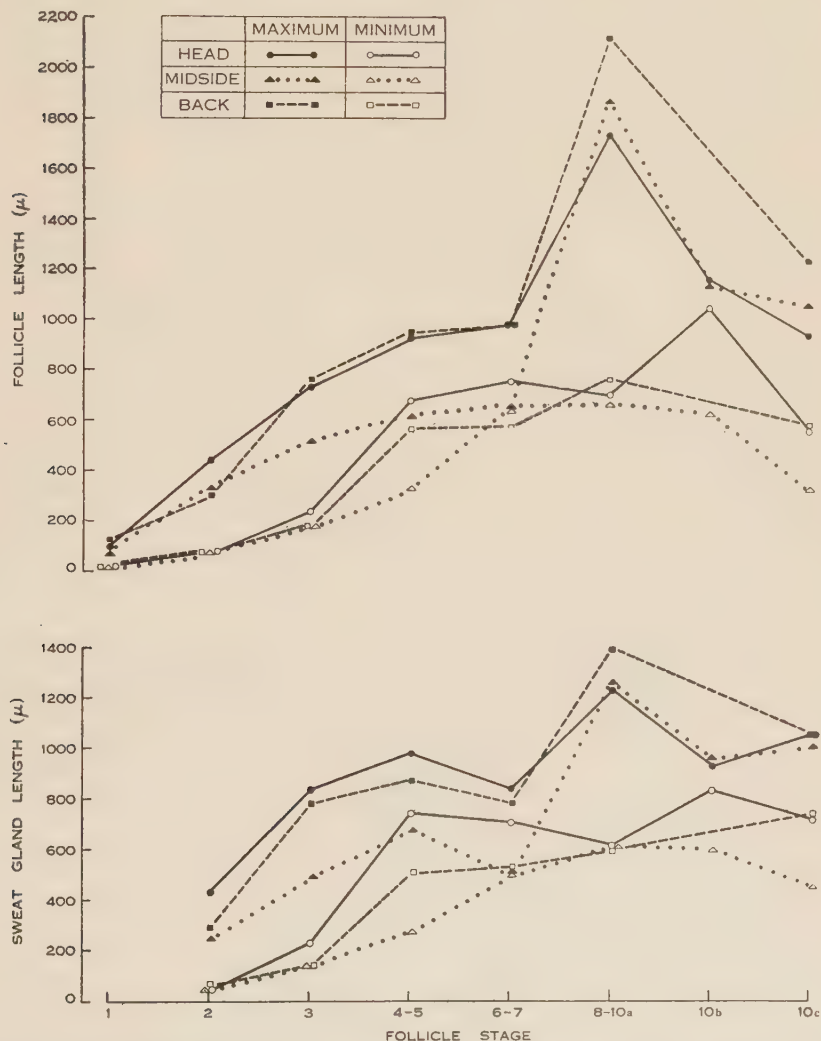


Fig. 5.—Relation between follicle stage, maximum and minimum follicle length, and sweat gland length in *B. indicus*  $\times$  *B. taurus*.

(b) *Development of the Follicle and Hair Population*

(i) *Follicle Density*.—Observations on the density of the follicle and hair population are summarized in Table 4 and illustrated in Figure 7. Up to the 141st day of gestation the follicle density is greatest on the head; during this period there



is little difference in density between the two trunk positions. Follicle density on the head rises relatively sharply to a peak (about 194 per  $\text{mm}^2$ ) at the 141st day, but thereafter falls slowly throughout the rest of the period studied. In the oldest foetus (263 days) the total number of follicles on the head is of the order of 42 per  $\text{mm}^2$ .

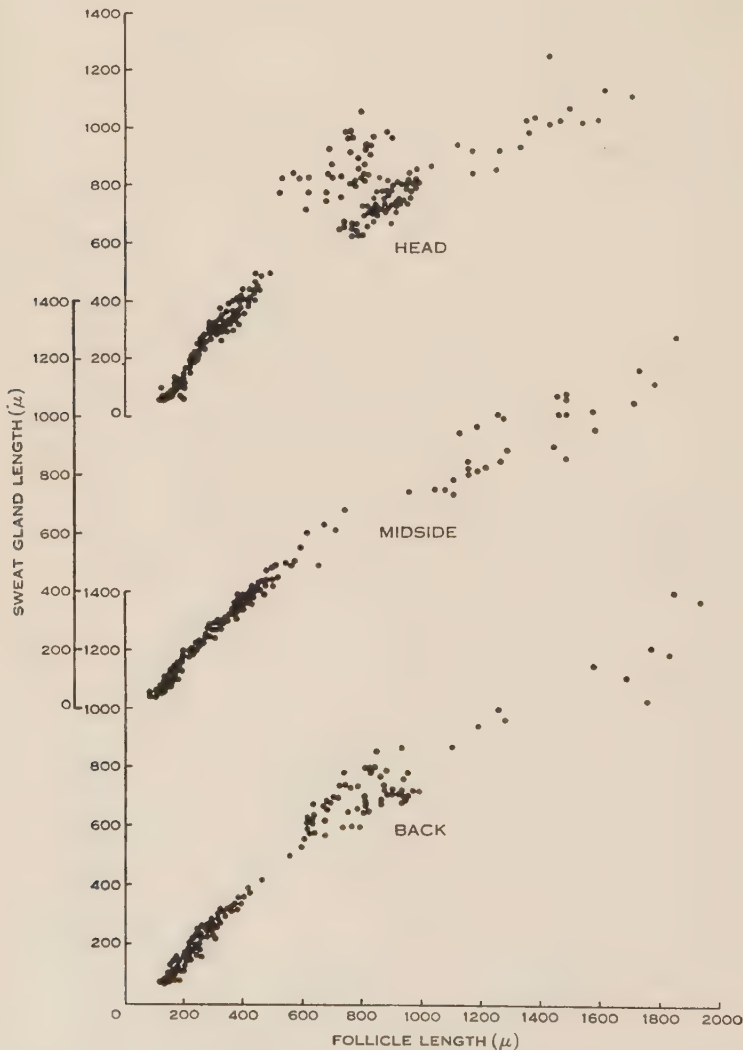


Fig. 6.—Relation between sweat gland length and individual follicle length in *B. indicus*  $\times$  *B. taurus*. Many of the points are too numerous to be shown separately.

On the midside (later samples shown in Table 4) the follicle density reaches a peak of about 355 per  $\text{mm}^2$  at about the 180th day, and then falls, rapidly at first and later more slowly, to approximately 120 per  $\text{mm}^2$  at the 263rd day.

Because the maximum observed follicle density on the back occurs in a specimen (B67) in which the least advanced follicles are at stage 2a, the peak density in this region must occur earlier; i.e. approximately 160 days. From about 160–263 days the density on the back is approximately intermediate between the head and the midside.

Since the peak of follicle density must occur at or before the end of follicle initiation (i.e. 141 days on the head region; 160–180 days on the trunk positions), after this time the follicle density must be inversely proportional to the increase in surface area. The approximate change in skin area deduced from foetal body weight raised to the power of 0.67 is plotted in Figure 7.

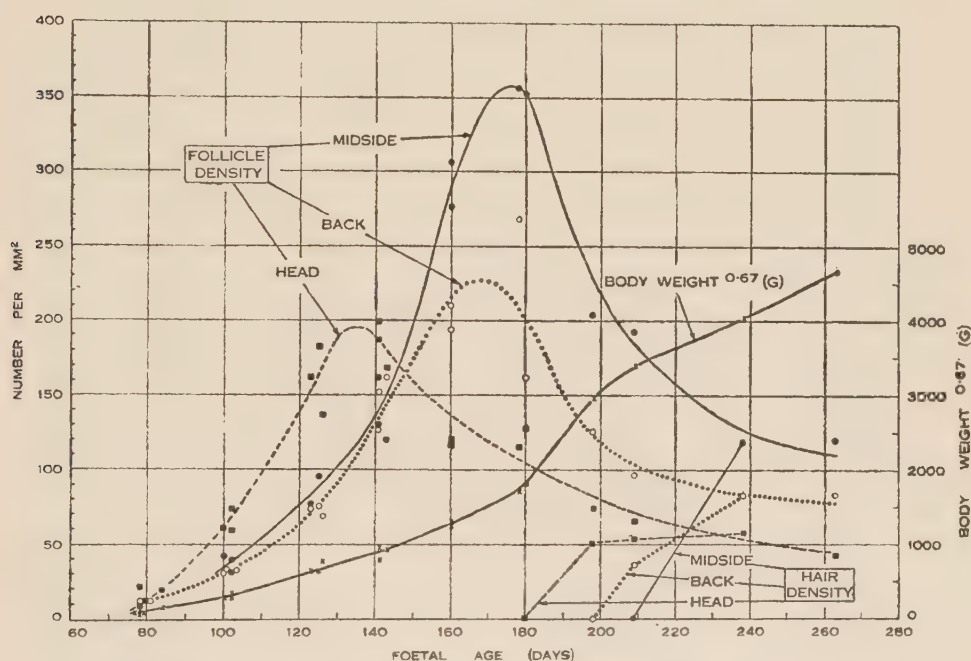


Fig. 7.—Relation between foetal age and follicle and hair density (later skin samples only included) on the head, midside, and back in *B. indicus* × *B. taurus*. The curves through the follicle density points have been drawn by freehand. The index of skin area is given by body weight raised to the power 0.67 (g).

Table 4 shows that from 160 to 263 days on the midside the number of follicles per mm<sup>2</sup> in the initial samples is markedly less than in the later samples. This difference is possibly due to shrinkage of the skin in this region when the foetus is fixed entire. No difference can be detected between the follicle densities of the initial midside samples of the crossbred material and those of similar samples of *B. taurus* (Lyne and Heideman 1959). Later samples from the *B. taurus* material have not been examined.

(ii) *Hair Density*.—In *B. indicus* × *B. taurus* hairs were first seen on the head at 198 days; on the trunk, follicles on the back contained keratinized hairs before



those on the midside. By 238 days all follicles on the head and back, and nearly all on the midside, had grown keratinized hairs.

(e) *Skin Thickness in B. indicus* × *B. taurus* and *B. taurus*

Macroscopic measurements of the thickness of skin samples from *B. indicus* × *B. taurus* and *B. taurus* clearly show that the total skin thickness increases from about the 80th day, when hair follicle initiation begins to birth (Fig. 8). In both *B. taurus* and the crossbreds the skin on the head is distinctly thicker than that on

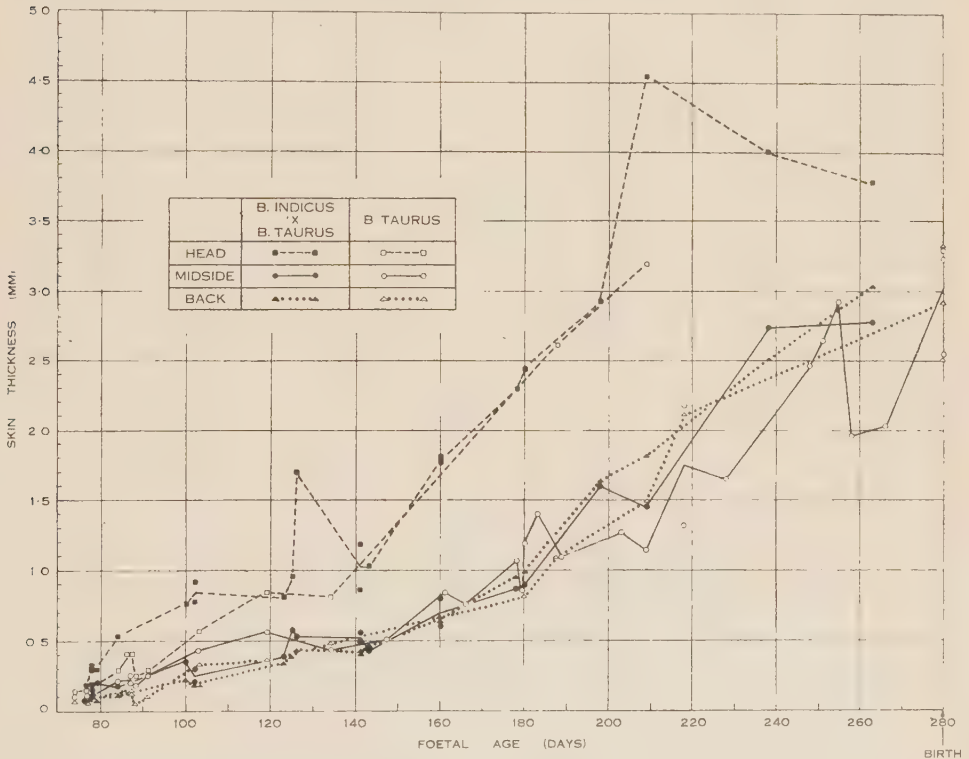


Fig. 8.—Relation between the foetal age and skin thickness on the head, midside, and back in *B. indicus* × *B. taurus* and *B. taurus*.

the two trunk positions. No difference can be detected between the midside and back. Because of the limited amount of material available none of the variations between individual animals can be attributed to difference between breeds.

#### IV. DISCUSSION

The slower rate of development of the first-formed follicles compared with the later- and last-formed follicles in the three positions (Table 2) was also found on the midside of *B. taurus* (Lyne and Heideman 1959). In other mammals—for example, in the bandicoot *Perameles nasuta* (Lyne 1957)—the rate of development of the first-formed follicles is also much slower than it is for the last-formed follicles,



excluding the follicles which develop by branching. In the brush-tailed possum *Trichosurus vulpecula* (Lyne, unpublished observations), however, the rate of development of the first-initiated follicles is much greater than it is for follicles formed later. Also, it is of interest to note that in the bovine and bandicoot, the first-formed follicles, which always develop relatively slowly, are the largest while the last-formed follicles are much smaller and develop at a faster rate. By contrast, in *Trichosurus*, the first-formed follicles are very small—so small in fact, that their hairs, about 0.5 mm in total length, project only 0.25 mm above the epidermis, and they develop very rapidly. From this evidence it is concluded that the rate of follicle development, at least in these species, is closely related to mature size of the follicle.

To our knowledge, very little has been published on the rate of development of the different types of follicles in other species. In the Merino sheep there is no marked difference in the mature size of the different follicle types but some of the smallest follicles (derived secondaries) appear to develop at the fastest rate (Hardy and Lyne 1956). The slower rate of development of large pelage follicles compared with small ones is possibly a general mammalian feature. Vibrissal follicles, however, appear to develop more rapidly than pelage follicles. In the mouse (Davidson and Hardy 1952) the vibrissal follicles, which are larger and stouter than those of the pelage, develop from stage 1 to stage 8 in approximately 6 days compared with 9 days for the most advanced pelage follicles. In addition to being larger, all vibrissal follicles possess blood sinuses and abundant nerve endings which distinguish them from pelage hair follicles.

The rate of development of the most advanced first-formed follicles in *B. indicus*  $\times$  *B. taurus* is very similar on the head and trunk positions despite differences in follicle density.

Comparison of Figures 1(b) and 7 shows that the cessation of follicle initiation and the peaks of follicle density are approximately contemporaneous, and on the head region this occurs 40 days earlier (140 days gestation) than on the midside. The greater density of the follicles on the midside region after 150 days is probably due to the formation of larger follicle groups; the midside maintains a higher density than the other regions, at least up to birth.

In the midside region, the rate of development of the different follicle types in the crossbreeds is practically identical with that found in *B. taurus* (Lyne and Heideman 1959). Although the follicle densities are similar in foetuses of the two breed types they are distinctly different in the adult animal. For example, in various European breeds (*B. taurus*) the follicle densities in mature animals in good condition range from 733 to 1111 per cm<sup>2</sup> (Carter and Dowling 1954; Dowling 1955; Nay and Hayman 1956). In contrast, the mean number of follicles per cm<sup>2</sup> is 1321 in mature Zebu crossbreeds (*B. indicus*  $\times$  *B. taurus*) (Dowling 1955), and 1507–1698 in mature Zebus (*B. indicus*) (Dowling 1955; Nay and Hayman 1956). Dowling found highly significant differences in hair follicle population between *B. indicus* and *B. taurus* species of cattle after allowing for the regression of follicle density on heart girth. Further studies are needed in order to determine why the follicle density declines to the lowest level in *B. taurus*.

The studies reported in this paper, and in a previous paper (Lyne and Heideman 1959), are necessary as an initial step to further qualitative and quantitative investigations of the integument of different breeds of cattle.

#### V. ACKNOWLEDGMENTS

The authors are particularly indebted to Mr. J. F. Kennedy, Officer-in-Charge, National Cattle Breeding Station, "Belmont", Rockhampton, Qld., for all the material of *B. indicus* × *B. taurus*. Also, we wish to thank Mr. W. H. Clarke and Mr. E. W. Taylor for technical assistance and Mr. M. R. F. Blair for preparing the figures for publication.

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## SHORT COMMUNICATION

### COMPLEXES OF COPPER WITH SOME PYRROLIZIDINE ALKALOIDS AND WITH SOME OF THEIR ESTERIFYING ACIDS\*

By K. J. FARRINGTON† and C. H. GALLAGHER†

Bull *et al.* (1956) and Bull and Dick (1959) have shown that the repeated intake of pyrrolizidine alkaloids of the plant *Heliotropium europaeum* can lead to severe liver damage, and to the abnormal accumulation of copper in the livers of sheep and rats.

The possibility of complex formation between copper and pyrrolizidine alkaloids was investigated in the present study.

#### *Methods and Results*

(i) *Complexes at Alkaline pH.*—2N NaOH was added to a series of solutions of pyrrolizidine alkaloids in aqueous  $\text{CuSO}_4$  until pH 12 was reached.  $\text{Cu}(\text{OH})_2$  was allowed to settle, and was then centrifuged off. The colour of the supernatants was compared with a solution of  $\text{CuSO}_4$  brought to pH 12 with 2N NaOH. A characteristic dark blue colour was detectable over a wide range of proportions of alkaloids to  $\text{CuSO}_4$  in the case of those alkaloids which were esters of an organic acid which had two or more hydroxyl groups on adjacent carbon atoms (Table 1).

Solutions of the free esterifying acids, lasiocarpic, trachelanthic, and viridifloric acids, all of which contain an  $\alpha$ -glycol group, gave the characteristic colour with  $\text{Cu}^{++}$  at alkaline pH, but a solution of heliotric acid, which does not have an  $\alpha$ -glycol group, did not give the colour.

(ii) *Molecular Proportions of the Complexes at Alkaline pH.*—Solutions of different concentrations of the alkaloids lasiocarpine, lasiocarpine *N*-oxide, and monocrotaline or of lasiocarpic and trachelanthic acids were prepared, and the same amount of  $\text{CuSO}_4$  added to each. NaOH was added to pH 12 for the alkaloids and to pH 10 for the acids, and the solutions were adjusted to the same volume with water and filtered. Extinction coefficients at 640  $\text{m}\mu$ , the approximate absorption maximum of all the filtrates, were read against a blank which originally contained the same amount of  $\text{CuSO}_4$  and which was treated in the same way.

It was found by plotting the extinction coefficients that monocrotaline, lasiocarpine, lasiocarpine *N*-oxide, and trachelanthic acid combine with copper in the molar proportion of 2 : 1, and that lasiocarpic acid and copper appear to combine in the ratio of 1 : 1. The amount of copper involved in complex formation with lasiocarpic acid may, however, be exaggerated by salt formation with the carboxylic acid group which is not free in the alkaloid.

\* Manuscript received April 12, 1960.

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(iii) *Effect of pH on Complex Formation.*—When a solution of equimolar proportions of lasiocarpic acid and  $\text{CuCl}_2$  was titrated with  $0.1N$   $\text{NaOH}$ , the extinction coefficient at  $640\text{ m}\mu$ , read against a water blank and corrected for increases in

TABLE I  
COLOUR TEST WITH ALKALOIDS AND CUPRIC IONS AT ALKALINE pH

Alkaloid	Result	No. of Hydroxyl Groups in Esterifying Acid	Alkaloid	Result	No. of Hydroxyl Groups in Esterifying Acid
Echimidine	Positive	3*	Heliotrine	Negative	1
Echinatine	"	2*	Heliotrine <i>N</i> -oxide	"	1
Heliosupine	"	3*	Platyphylline	"	1
Lasiocarpine	"	2*	Senecionine	"	1
Lasiocarpine <i>N</i> -oxide	"	2*	Seneciphylline	"	1
Supinine	"	2*	Spectabiline	"	1
Supinine <i>N</i> -oxide	"	2*			
Monocrotaline	"	2*			

\* On adjacent carbon atoms.

volume, was found to increase as the pH rose through the range 4–9 approximately (Fig. 1), indicating that complex formation began at about pH 4.

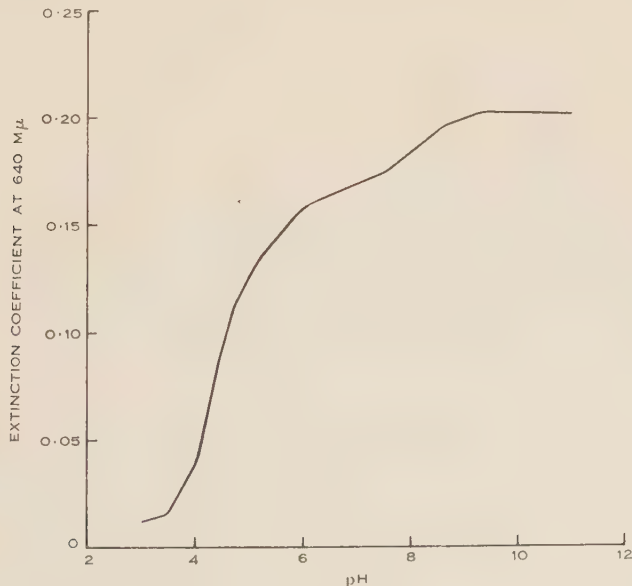


Fig. 1.—Titration of an equimolar solution of lasiocarpic acid and  $\text{CuCl}_2$  with  $0.1N$   $\text{NaOH}$ .

Further evidence on the formation of organic acid-copper complexes was obtained by following the change in pH of solutions, which resulted from the



addition of NaOH. Figure 2 shows how the mixtures of lasiocarpic acid and  $\text{CuCl}_2$ , heliotric acid and  $\text{CuCl}_2$ , and trachelanthic acid and  $\text{CuCl}_2$ , in the molar ratios of 1 : 1, 2 : 1, and 2 : 1 respectively, altered the pH titration curves from those resulting from titration of the acids and  $\text{CuCl}_2$  separately. Clearly heliotric acid, also, combined with  $\text{CuCl}_2$ .

(iv) *Copper Complexes at pH 6*.—When alkaline solutions of alkaloids and copper were treated with acid to lower the pH, precipitation occurred between pH 9 and pH 6. Consequently it was not possible to study complex formation between copper and the complete alkaloids at pH 6.

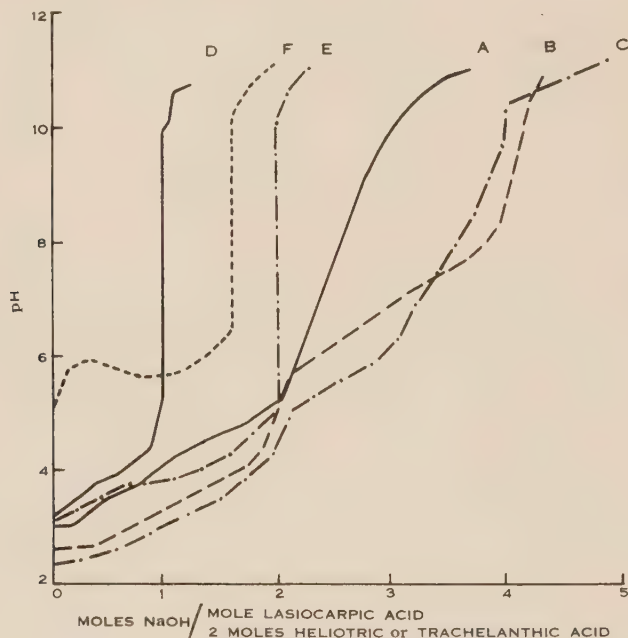


Fig. 2.—Titration with 0.1N NaOH of: *A*, lasiocarpic acid (100 mg) and  $\text{CuCl}_2$ , molar ratio 1 : 1; *B*, heliotric acid (100 mg) and  $\text{CuCl}_2$ , molar ratio 2 : 1; *C*, trachelanthic acid (100 mg) and  $\text{CuCl}_2$ , molar ratio 2 : 1; *D*, lasiocarpic acid (100 mg); *E*, heliotric or trachelanthic acid (100 mg); *F*,  $\text{CuCl}_2$ —same amount as for *B*.

Precipitation did not occur from solutions of the organic acids and copper at pH 6 and so the formation of acid-copper complexes at this pH was investigated. Solutions of lasiocarpic, trachelanthic, or heliotric acids and  $\text{CuSO}_4$  at pH 6 were blue-green in colour and absorbed light maximally between 710–760  $\text{m}\mu$ . Measurement of the extinction coefficients at the absorption maxima in solutions of different molecular proportions showed that at pH 6, lasiocarpic acid combines with copper in the molar ratio of 1 : 1, and that trachelanthic and heliotric acids combine with copper in the ratio 2 : 1. Precipitation occurred from solutions of heliotric acid and copper above pH 7, and with trachelanthic acid and copper above pH 9, on standing. No precipitation occurred with lasiocarpic acid and copper below pH 10.

*Discussion*

The alkaloids of *H. europaeum* which contain an esterified acid with an  $\alpha$ -glycol group, and the free  $\alpha$ -hydroxy acids of these alkaloids, have been found to form complexes with copper. Angelic acid, the only acid associated with the pyrrolizidine alkaloids of *H. europaeum* which does not have an  $\alpha$ -hydroxy group, was not tested. In view of the abnormal accumulation of copper in the liver of sheep which have grazed *H. europaeum*, and the similar accumulation associated with the administration of pyrrolizidine alkaloids to rats, these complexes may be of biological interest. It is not possible, on present published information, to determine whether liver damage by the alkaloids is a prerequisite for abnormal accumulation of copper. It may be that copper is accumulated as a result of complex formation either with the alkaloids or with the free acids after *in vivo* hydrolysis, and it may be purely chance that increased levels of copper were found only after liver damage was evident (Bull and Dick 1959).

*Acknowledgment*

We wish to thank Dr. C. C. J. Culvenor, Chemical Research Laboratories, C.S.I.R.O., for suggesting many of the experimental approaches, for valuable discussions, and for supplying the pyrrolizidine alkaloids and derivative compounds used in this study.

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